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13. ABSTRACT (Maximum 200) Growth factors and their receptors are crucial in the regulation of breast cancer cell growth. Since the HER-2 growth factor receptor pathway is implicated in the progression of breast cancer in the clinic, we have targeted HER-2 receptors for therapeutic intervention. Our research achievements / aims include: 1) Induction of breast tumor remission with antibody to HER-2 receptor in combination with chemotherapeutic drugs. A therapeutic advantage of antibody to HER-2 receptor combined with drugs that damage breast cell DNA is evident from initial studies. Antibodies to HER-2 receptor are undergoing testing with cisplatin and alkylating drugs to assess optimal treatment conditions for use in clinical trials. 2) Assessment of the clinical significance of HER-2 expression in resistance to DNA-damaging drugs. Reduced DNA repair is found on activation of HER-2 receptor by anti-HER-2 antibody. Investigation of DNA repair using a reporter gene and direct measure of drug sensitivity in breast cancer cells is ongoing. As compared to controls, HER-2-overexpressing tumors show more rapid regrowth after chemotherapy. 3) Defining the role of HER-2 and heregulin gene expression in antiestrogen resistance. Ligand for activation of HER-2/HER-3 receptor dimers, heregulin, and tumor cells bioengineered for production of heregulin are in use to determine effects of autocrine activation of HER-2 receptor on sensitivity to antiestrogens. Studies with HER-2-overexpressing breast cancer cells are also in progress.							
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INTRODUCTION

Breast cancer accounts for 32% of all cancers in women. Despite some limited success in therapeutic intervention, new approaches in breast cancer therapy are needed. We are working to target growth factor receptor pathways which regulate breast cancer cell growth. HER genes encode for epidermal growth factor (EGF), HER-2, HER-3 and HER-4 growth factor receptors. One or more of these oncogenes is over-expressed in two-thirds of human breast cancers. Overexpression of EGF and HER-2 gene products is associated with poor clinical outcome and appears to predict the clinical response to chemotherapy. Antibodies to HER receptors are known to have a cytostatic effect in suppressing growth of cells with HER gene overexpression. New data from our laboratory indicate that activation of growth factor receptors by anti-HER-2 receptor antibody can enhance the sensitivity of cells to drugs that damage DNA and, thereby, potentiate their cytotoxic effect (16, 19). We are encouraged that, due, in part, to the latter work, humanized monoclonal antibody to HER-2 receptor is now undergoing phase III clinical trials at UCLA and other major research centers for therapy of patients with metastatic breast cancer (15, 20).

In order to establish a firm biologic basis for these important preclinical and clinical observations, human breast cell lines with well-characterized HER-2 expression have been bioengineered in our laboratory. These model cell lines are in use for study of the therapeutic utility of a newly-purified ligand to heterodimers of HER-2/HER-3 receptor, heregulin, and humanized monoclonal antibody to HER-2 receptor for human application (18). Our ongoing research efforts are aimed at the following:

Task 1) Testing of the antitumor effects of humanized monoclonal antibody to HER-2 receptor in combination with chemotherapeutic drugs (cisplatin and alkylators) that damage cellular DNA. The therapeutic benefit of combined therapy with antireceptor antibody and cytotoxic drugs is being investigated, with aims to optimize conditions for maximal cytotoxic effects. HER-2 antibody-induced signal leading to blockade of DNA repair appears to provide a biologic basis for increased killing of breast cancer cells after exposure to DNA-damaging agents. These findings are now being applied in the clinic at UCLA, with ongoing Phase III trials to assess the use of HER-2 antibody and DNA-damaging drugs in patients with advanced breast cancer.

Task 2) Clinical significance of HER-2 overexpression in drug resistance. To test the role of HER-2 in genesis of chemotherapy resistance, parental cells with low-expression of HER-2 and bioengineered daughter cells with high-expression of HER-2 are being compared for relative drug sensitivity. This approach may allow more accurate prediction of chemotherapeutic drug response and, thereby, lead to better choice of treatment strategies in affected patients.

Task 3) Role of HER-2 and heregulin gene expression in antiestrogen resistance. The hypothesis that heregulins may be a class of estrogen-induced growth factors and/or modulate estrogen receptor pathways via HER-2 receptor will be tested. New strategies for reversal of endocrine treatment failure in breast cancer can derive from this work.

Please note that Tasks 1-3 above incorporate all the components of Tasks 1-5 in the original research proposal. With the progression of our research work, we have found that the outline above is a more coherent and logical description of our laboratory efforts. In brief, Task 1 corresponds to Tasks 2, 3 and 5 in the original proposal; Task 2 corresponds to Task 4a-c in the original proposal; and Task 3 corresponds to Tasks 1 and 4d in the original proposal (see Addendum A. below). _

EXPERIMENTAL RESULTS

Since the start of this project, substantial progress has been made in studies of the therapeutic advantage of treatment with humanized monoclonal antibody to HER-2 receptor (rhuMAb HER-2) and the chemotherapeutic drug, cisplatin. As noted above, this work has also enabled the start at UCLA of Phase III clinical trials of humanized monoclonal antibody to HER-2 receptor with and without chemotherapy in patients with metastatic breast cancer. Completion of the studies below is required to continue this effort.

Task 1) To induce breast tumor remission with antibody to HER-2 receptor in combination with chemotherapeutic drugs.

Approximately 30% of human breast cancers have amplification and/or overexpression of HER-2 gene which encodes a cell surface growth-factor receptor. Overexpression of this receptor, HER-2, is associated with poor outcome and may predict the clinical response to chemotherapy (5,16,17). We have confirmed earlier observations showing that monoclonal antibodies to HER-2 receptor have a cytostatic effect in suppressing the growth of breast cancer cells with overexpression of HER-2 gene product. In order to elicit a cytoidal effect, therapy with antireceptor antibody was used in combination with the DNA-damaging drug, cisplatin, and this combined treatment produced a synergistic decrease in cell growth which was significantly different from the effects of either antibody or cisplatin given alone ($P<0.001$; 17). Further experiments with antireceptor antibody in combination with doxorubicin also show a significant therapeutic advantage in this combined therapy as compared to controls ($P<0.01$), but the doxorubicin-antibody combination may have less antitumor activity than the cisplatin-antibody combination (19).

Cyclic therapy regimens are commonly used in the clinic. On testing the use of repeated, cyclic doses of cisplatin in combination with rhuMAb HER-2, we found a more profound anticancer effect (19). Of mice receiving cisplatin or rhuMAb HER-2 alone, mean tumor volumes compared to control were reduced moderately over a 2-month treatment period ($P<0.001$), but no tumor remissions were observed. In contrast, over a 2-month treatment period, combined cisplatin-antibody therapy produced a marked reduction in tumor volumes as compared to controls ($P<0.001$). All animals that received both rhuMAb HER-2 and cisplatin had tumor remission after 2-3 cycles of therapy, with complete remission in 83% and partial remission in the remaining animals. Effects of cisplatin-antibody therapy were significantly different from those found with antibody treatment alone ($P<0.005$; 19). These data show marked cytotoxicity of cisplatin when given with rhuMAbHER-2 and show therapeutic value in treatment with these agents in a cyclic combination regimen as is commonly used in clinic.

To evaluate the potential influence of schedule of administration of rhuMAb HER-2 when combined with cisplatin on the growth of HER-2-overexpressing MCF-7 cells, cells were implanted subcutaneously in estrogen-primed female athymic mice for 14 days and then randomized to one of 18 treatment groups. Study groups included control (human IgG1), IgG1 with cisplatin, rhuMAb HER 2 alone, and rhuMAb HER-2 with cisplatin, with several alternate times of administration for drug and antibody (refer to study design in Addendum B. below). A summary of the data is shown in Figure 1 below. The effect of rhuMAb HER-2 given at various times before or after cisplatin on breast tumor volume was compared to control groups, and tumor nodules were monitored to day 21 (19). In these experiments, rhuMAb HER-2 was given at 3 mg/kg, and cisplatin was used at a dose of 0.5 mg/kg. MAb HER-2 was injected on day 1 (time 0 or after 8 hrs), day 2 (24 hrs), day 3 (72 hrs) or day 5 (120 hrs), with the antitumor effect compared to IgG control given at day 1. All treatments with antibody alone elicited a significant growth suppression as compared to control ($P<0.05$). Cisplatin at 0.5 mg/kgwas administered with IgG at days 1 (time 0 or after 8 hrs), day 2 (24 hrs), day 3 (72 hrs) or day 5 (120 hrs). Therapy with cisplatin on the several days tested also blocked tumor formation in athymic mice as compared to the IgG control group ($P<0.05$). Several different combination treatments with antibody and drug were then done. MAb HER-2 was given on day 1 (see time 0 in Figure 1), with cisplatin administration varying from day 1 (time 0 or 8 hrs) through day 5 (120 hrs). Each of these treatment protocols promoted significant growth suppression as compared to the IgG control group ($P<0.01$). With the exception of groups in which cisplatin followed antibody by 3 to 5 days, these treatment groups exhibited significantly more tumor growth inhibition than mice treated with cisplatin alone ($P<0.05$). However, cisplatin followed by rhuMAb HER-2 at days 2 to 5 did not elicit greater tumor growth suppression than antibody given alone on corresponding days ($P>0.25$). Moreover, administration of rhuMAb HER-2 at 1-4 days after cisplatin showed less antitumor efficacy than those regimens in which antireceptor antibody preceded cisplatin ($P<0.05$). These data demonstrate that the order of antibody-cisplatin administration is critical and clearly affects the magnitude of observed antitumor responses in HER-2-overexpressing human breast cancer xenografts (19). Hence, it is apparent that the schedule and timing of therapeutic agents will be important in achieving synergistic killing of tumor cells in the clinic. These data may also prove useful in further elucidating the biologic basis of drug-antibody synergy.

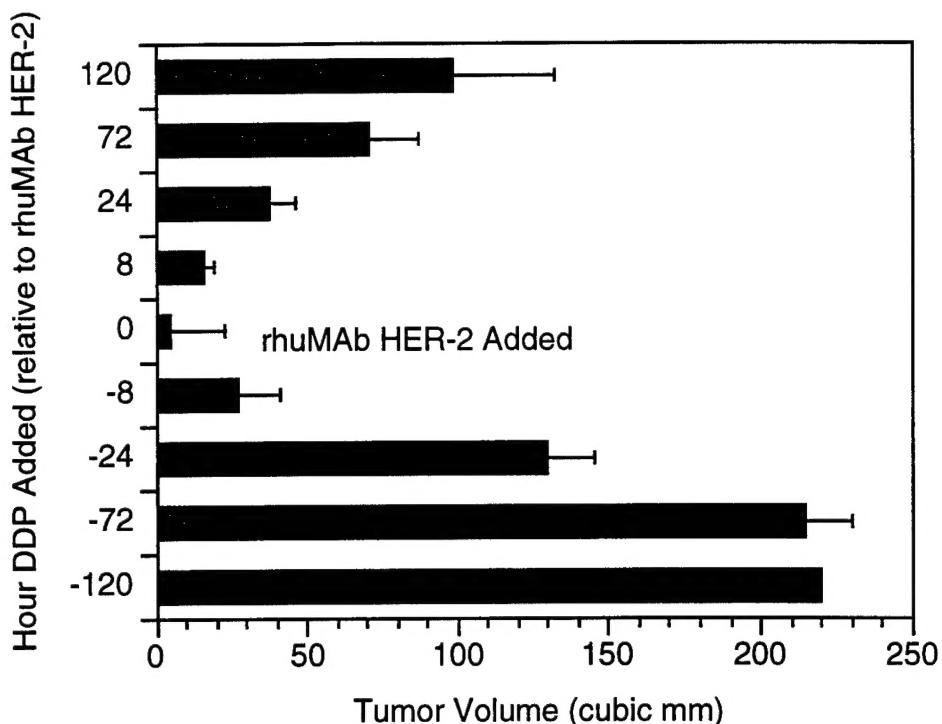


Figure 1. Administration of cisplatin (DDP) within 8 hours of rhuMAb HER-2 treatment time is crucial for achieving maximal antitumor effect in human breast cancer cells with HER-2 overexpression. In experiments shown here using a nude mouse tumor xenograft model, treatment with rhuMAb HER-2 was at hour 0, and DDP was given up to 120 hrs before or after antibody dosing as shown on the ordinate axis. Synergy was optimal if DDP and rhuMAb HER-2 were given at the same time (see hour 0) or within an 8 hr time period (-8 to +8 hrs). No drug-antibody synergy occurred if DDP preceded rhuMAb HER-2 by 72 to 120 hrs. See text for additional details .

Task 2) To assess the significance of HER-2 expression in resistance to DNA-damaging drugs.

To evaluate the mechanism for antibody-drug synergy, DNA repair (unscheduled DNA synthesis) was measured in cancer cells using incorporation of [³H]thymidine and autoradiography, and formation and repair of cisplatin-induced DNA adducts was also measured. Treatment with cisplatin led to a marked, dose-dependent increase in unscheduled DNA synthesis which was significantly reduced by combined treatment with antireceptor antibody in HER-2-overexpressing cells ($P<0.001$). Therapy with antibody to HER-2 receptor also led to a 35-40% reduction in repair of cisplatin-DNA adducts after cisplatin exposure and, as a result, promoted drug-induced killing in target cells (17). This phenomenon may provide a rationale for more selective targeting of overexpressed growth factor receptors in cancer cells.

As noted above, clinical findings suggest that overexpression of HER-2 gene may be related to chemotherapy resistance. The role in this phenomenon of ligand or ligand overexpression leading to activation of HER-2 signal pathway is not known. Binding of certain growth factors to their cognate receptors has been reported to modulate cell sensitivity to drugs, but the role of heregulin (HRG), a ligand leading to HER-2 receptor activation, in drug resistance remains to be tested. We have begun to compare the growth properties and drug sensitivity of MCF-7 cells with no expression of HRG to that of paired MCF-7 cells with high HRG expression (18). Cells with low-expression of HER-2 and daughter cells with high-expression of HER-2 are being tested for drug sensitivity as influenced by HRG. This work will provide data on the biological role of HER-2 oncogene in the genesis of drug resistance found in human cancers, and may allow future design of

simple tests to predict biologic response to chemotherapy in affected patients. In addition, clarification of the role of HRG may lead to use of ligand or, alternatively, an anti-HRG antibody in future clinical interventions.

Task 3) To define the role of HER-2 and heregulin expression in antiestrogen resistance.

Members of both steroid and peptide receptor classes are important prognostic factors in human breast cancer (5,17). Clinical data indicate that overexpression of the HER-2 gene is associated with an estrogen receptor-negative phenotype. We have demonstrated that introduction of a HER-2 cDNA, converting non-overexpressing breast cancer cells to those which overexpress this receptor, results in development of estrogen-independent growth that is insensitive to both estrogen and the antiestrogen, tamoxifen. Moreover, activation of HER-2 receptor in breast cancer cells by the peptide growth factor, heregulin, leads to direct and rapid phosphorylation of ER on tyrosine residues. This is followed by interactions between ER and estrogen-response elements in the nucleus and production of an estrogen-induced protein, progesterone receptor (PR). With long-term exposure to HRG, down-regulation of ER and, in turn, PR occurs, producing an ER- / PR-phenotype (18). Similarly, overexpression of HER-2 receptor in estrogen-dependent tumor cells promotes ligand-independent down-regulation of ER and a delayed autoregulatory suppression of ER transcripts (18). These data demonstrate a direct link between these two receptor pathways and suggest one mechanism for genesis of endocrine resistance in breast cancers.

FUTURE WORK

As detailed above, we have already made considerable progress in evaluating the anticancer efficacy of rhuMAb HER-2 alone and in combination with chemotherapeutic drugs. We plan to continue this effort in support of ongoing clinical trials before we become committed to therapeutic approaches which are not thoroughly tested on scientific grounds. Specific goals are outlined below:

Task 1) To induce breast tumor remission with antibody to HER-2 receptor in combination with chemotherapeutic drugs.

Cancer therapy requires new approaches which minimize toxicity to normal cells and maximize damage to tumor targets. Our work has demonstrated synergistic effects in tumor cell treatment with antireceptor antibody to HER gene products and cisplatin (1,7). RhuMAb HER-2 will also be tested in combination with an alkylating drug as representative of another class of chemotherapeutic drugs which damage cellular DNA. MCF-7 and MCF-7 HER-2 cells will be plated at a density of 10^5 cells/well in 96-well plates and allowed to adhere. Cells will then be exposed to MAb 4D5 or rhuMAb HER-2 alone or in combination with 4-hydroperoxycyclophosphamide, an activated form of cyclophosphamide for use in in vitro studies, or other alkylating agents, such as triethylenethiophosphoramide (thiotepa), at concentrations reported to have efficacy in prior studies (see 16). After 72 hours, the plates will be washed with PBS, stained with 0.5% crystal violet in methanol, and analyzed for relative cell proliferation as before (17,18). Human IgG1 will also be utilized as a control. Other in vitro assays, including soft agar colony formation, will also be utilized to test antibody-alkylator drug interactions. Nude mouse tumor formation studies will follow to assess promising drug-antibody combinations in vivo. The design of in vivo studies will be derived from these preliminary in vitro studies. Further experiments will follow the design described above as warranted.

Task 2) To assess the significance of HER-2 expression in resistance to DNA-damaging drugs.

A role of HER oncogenes in modulation of chemotherapeutic drug sensitivity is suggested from results of several clinical studies, and, if correct, could have important implications in patient management decisions. We have begun to directly compare drug sensitivity of parent cells with low expression of HER-2 and bioengineered daughter cells with high expression of HER-2 (16). These studies are being done in

vitro and in nude mouse models using our human breast cancer lines with cisplatin and other agents, including cyclophosphamide, doxorubicin, fluorouracil, methotrexate, paclitaxel, thiotepa and vinblastine. A full description of the experimental approach is provided elsewhere (16).

A spectrum of lesions is known to be induced in DNA by chemotherapeutic drugs (8,24).

Alkylating drugs generally promote covalent binding of alkyl groups to guanine bases in DNA, while cisplatin tends to produce intrastrand adducts and interstrand crosslinks in DNA. In cells resistant to DNA-damaging drugs, increased levels of DNA repair enzymes have been detected, while DNA repair-deficient cells exhibit markedly enhanced sensitivity to alkylating agents (24). It is notable that the tumor suppressor gene, p53, is likewise involved in the cellular response to DNA damage, with mutation of p53 leading to deficiency in the repair of damaged DNA (23). On the basis of observed increments in cell sensitivity to DNA-damaging drugs after antireceptor antibody treatment (2,11,18), we postulate that antireceptor antibody elicits blockade of DNA repair. Some potential pathways leading to suppression of DNA repair are outlined in Figure 2.

We will first assess DNA repair by measurement of unscheduled DNA synthesis (UDS), with autoradiographic (25) and biochemical (14) methods detailed before. Testing of antibody-dependent suppression of UDS will be extended to include anti-HER 2 receptor antibody with our additional cell lines with HER-2 overexpression in order to determine the generality of this phenomenon (19). Further testing with cisplatin and antibody in MCF-7 cells with HRG overexpression (17) will elucidate the influence of natural ligand in DNA repair pathways.

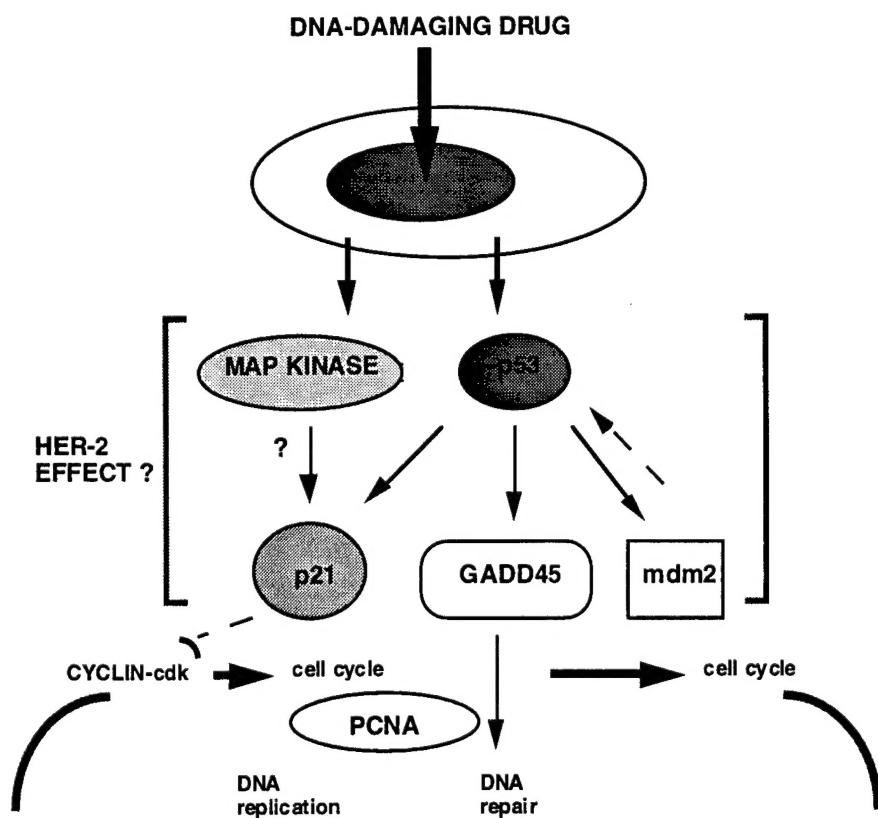


Figure 2. Potential pathways for HER-2 receptor-mediated blockade of DNA repair. Treatment of breast cells with a DNA-damaging drug normally activates p53 and p21, leading to cell cycle arrest and initiation of DNA repair for preservation of DNA integrity. However, pre-treatment of cells with antibody to HER-2 receptor elicits blockade of DNA repair after exposure to DNA-damaging drugs, leading to reduced DNA integrity and greater cell death. The pathway triggered by antireceptor antibody may interfere with p21 activity or that of other signal molecules involved in regulation of DNA repair (see MAP kinase, GADD 45, mdm2, PCNA in scheme above).

Quantitation of the formation and repair of cisplatin intrastrand adducts and interstrand crosslinks in purified DNA (6,7,13) in affected cells will provide another measure of DNA repair capability. An atomic absorption spectrometer, as well as an inductively-coupled plasma atomic emission spectrometer (ICP-AES), for sensitive determination of cisplatin content in cell DNA (9,13) is in use for these studies in the Division of Environmental Medicine at UCLA. Results of studies to measure the formation and repair of cisplatin-DNA adducts in C13 pRVHER-2 cells by atomic absorption spectrometry have been presented (17). Combination treatment of cells with cisplatin and rhuMAb HER-2, HRG or control solution would be tested to evaluate DNA repair activity. Results of our work demonstrate that removal of cisplatin adducts from the genome of HER-2-enriched cells is reduced 35-40% in the presence of antireceptor antibody (17).

Our prime objective in this work is to firmly establish the contribution of DNA repair in receptor-modulated sensitivity of cancer cells to DNA-damaging drugs. This would provide a strong rationale for pursuit of combined drug-antibody therapy in the clinic (2). Pending the satisfactory completion of this component, we will consider additional approaches to molecular mechanisms involved in this phenomenon (see Figure 1). Evaluation of initial steps in the signal transduction pathway from surface membrane to the interior of the cell (see 3, 4, 5, 12, 21, 22) and assay of p21, a critical modulator of cell cycle arrest prior to the onset of DNA repair (10), will be one course of investigation (19).

We are conducting a parallel series of studies with human heregulin genes transfected in human MCF-7 parent cells which have no native HRG transcripts (12,18). The MCF-7 cells with heregulin expression will be used to test for growth, cisplatin sensitivity, and DNA repair capability using established methods (17,18). An MCF-7 cell line transfected with full-length HRG-alpha (12) is also undergoing selection, thus allowing for comparison of the properties of α - vs β -HRG. Additional studies with HRG-producing MCF-7 cells *in vivo* will aid in evaluating the efficacy of HRG on growth and progression of breast cancer cells with a single-copy of HER-2 receptor. Sensitivity of MCF-7 pHRG cells to cisplatin and cisplatin-antibody therapy will also be tested in this *in vivo* model using methods as detailed above.

To test for changes in cellular sensitivity to chemotherapy drugs on treatment with HRG, an *in vitro* treatment strategy will be used first. Performance of *in vivo* studies of cisplatin sensitivity would depend on results of the latter studies. As warranted, HRG will be tested *in vivo* in experiments with MCF-7 cells.

Determination of the chemotherapeutic drug sensitivity of breast cancer cells with and without overexpression of HER-2 gene will be continued as noted above (also, cf.17,18). The potential role of HER oncogene in modulation of chemotherapeutic drug sensitivity has been suggested from results of several clinical studies (5,15), and, if correct, could have important implications in patient management and treatment decisions.

Task 3) To define the role of HER-2 and heregulin expression in antiestrogen resistance.

We have demonstrated that introduction of a HER-2 cDNA, converting non-overexpressing breast cancer cells to those which overexpress this receptor, results in development of estrogen-independent growth (18). In contrast to MCF-7 parental cells, MCF-7 HER-2 cells are insensitive to both estrogen and the antiestrogen, tamoxifen. Moreover, activation of the HER-2 receptor in breast cancer cells by the peptide growth factor, heregulin, leads to direct and rapid phosphorylation of estrogen receptor (ER) on tyrosine residues. This is followed by interaction between ER and the estrogen-response elements in the nucleus and production of an estrogen-induced protein, progesterone receptor (PR). With long-term treatment of breast cells with heregulin, a sustained down-regulation of ER and PR occurs, leading to an ER-/PR- phenotype. In addition, overexpression of HER-2 receptor in estrogen-dependent tumor cells promotes ligand-independent down-regulation of ER and delayed autoregulatory suppression of ER transcripts (18). These data demonstrate a direct link between these two receptor pathways and suggest one mechanism for development of endocrine resistance in human breast cancers. We plan to continue this work using other HER-2-overexpressing breast cancer cells to assess the generality of the findings. In addition, ligand for activation of HER-2 receptor, heregulin, and tumor cells bioengineered for production of heregulin will also be used to determine effects of autocrine/paracrine activation of HER-2 receptor on sensitivity to antiestrogens.

CONCLUSIONS

In summary, substantial progress has been made in studies of the therapeutic advantage of treatment with humanized monoclonal antibody to HER-2 receptor and chemotherapeutic drugs. This preclinical work has enabled the initiation of unique Phase III clinical trials of humanized monoclonal antibody to HER-2 receptor with and without chemotherapy in patients with metastatic breast cancer (15, 20). Continuation of our laboratory studies is required to promote further progress in this clinical effort at UCLA and other clinical research centers. We hope that elucidation of the molecular mechanism underlying the synergistic effect of cisplatin and antireceptor antibody will promote further progress in this new therapeutic initiative. We thank you for your continued support of this work.

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ADDENDUM

A. STATEMENT OF WORK (original version)

Task 1, Dose and schedule of humanized monoclonal antibody to HER-2 receptor on growth of human breast cells, Months 1-6.

- a) In vitro studies of proliferation, colony formation, DNA synthesis, Current & Months 1-4.
- b) In vivo nude mouse studies with human breast cells, Months 4-6.

Task 2, Conditions for maximal tumor cell killing by cisplatin in combination with monoclonal antibody to HER-2, Months 1-30.

- a) Quantitative analyses of dose-effect relationships in vitro and in vivo, Months 1-24
- b) Schedule and timing of cisplatin/antibody therapy, Months 20-30.

Task 3, Sensitivity of cells with overexpression of HER-2 to chemotherapeutic drugs, Months 18-48.

- a) Effect of HER-2 gene overexpression on drug sensitivity in vitro and in vivo, Months 18-30.
- b) Biologic basis of drug/antibody synergy via measure of DNA repair, Months 18-24.
- c) Basis of drug/antibody synergy via cisplatin-DNA adduct formation, Months 24-48

Task 4, Impact of HER-2 gene expression on cell sensitivity to estrogen and antiestrogens, Months 1-48.

- a) In vitro and in vivo evaluation of antiestrogen sensitivity in cells with and without HER-2 overexpression, Months 18-24.
- b) Estrogen-dependence of human breast cell growth in ovariectomized, athymic mice with and without estrogen supplementation, Months 18-24
- c) Effects of heregulin- β 1, a candidate ligand to HER-2 receptor, on modulation of estrogen receptor pathways in human breast cancer cells, Months 1-48.
 - i) In vitro study of specific binding of heregulin, estradiol and progesterone, Months 1-12.
 - ii) In vivo nude mouse studies, Months 12-48.

Task 5, Conditions for maximal breast tumor cell killing by alkylating agents (cyclophosphamide, thiotepa) in combination with humanized monoclonal antibody to HER-2 receptor in vitro and in vivo, Months 30-48.

B. Effect of order of administration of rhuMAb HER-2 and cisplatin on growth of HER-2-overexpressing human breast tumor xenografts in athymic mice

Group ^a	Test Agents ^b	Injection Time ^c	Dose ^d
1	Control IgG ^e	Day 14	3
2	rhuMAb HER-2	Day 14	3
3	rhuMAb HER-2	Day 15	3
4	rhuMAb HER-2	Day 17	3
5	rhuMAb HER-2	Day 19	3
6	Control IgG/Cisplatin ^f	Day 14	0.5
7	Control IgG/Cisplatin	Day 15	0.5
8	Control IgG/Cisplatin	Day 17	0.5
9	Control IgG/Cisplatin	Day 19	0.5
10	rhuMAb HER-2/Cisplatin	Day 14 / Day 14	3 / 0.5
11	rhuMAb HER-2/Cisplatin	Day 14 / Day 14 + 8h	3 / 0.5
12	rhuMAb HER-2/Cisplatin	Day 14 / Day 15	3 / 0.5
13	rhuMAb HER-2/Cisplatin	Day 14 / Day 17	3 / 0.5
14	rhuMAb HER-2/Cisplatin	Day 14 / Day 19	3 / 0.5
15	Cisplatin /rhuMAb HER-2	Day 14 / Day 14 + 8h	0.5 / 3
16	Cisplatin /rhuMAb HER-2	Day 14 / Day 15	0.5 / 3
17	Cisplatin /rhuMAb HER-2	Day 14 / Day 17	0.5 / 3
18	Cisplatin /rhuMAb HER-2	Day 14 / Day 19	0.5 / 3

- a Five mice per group. Cells were grown in estrogen-primed female athymic mice for 14 d and randomized to 18 treatment groups.
- b Order of injections are shown when both test agents are given. Doses of antibody were administered as indicated at various times before or after cisplatin.
- c Time of dosing defined from date of tumor innoculation.
- d Agent given as mg/kg mouse body weight, with all agents given as intraperitoneal injections.
- e Nonspecific human IgG1.
- f Control IgG dose (3 mg/kg) precedes cisplatin by 1 min.
- g RhuMAb HER-2 dose precedes cisplatin by 1 min.



HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells

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Growth of human breast cells is closely regulated by steroid hormone as well as peptide hormone receptors. Members of both receptor classes are important prognostic factors in human breast cancer. Clinical data indicate that overexpression of the HER-2 gene is associated with an estrogen receptor-negative phenotype. In this study, we demonstrate that introduction of a HER-2 cDNA, converting non-overexpressing breast cancer cells to those which overexpress this receptor, results in development of estrogen-independent growth which is insensitive to both estrogen and the antiestrogen, tamoxifen. Moreover, activation of the HER-2 receptor in breast cancer cells by the peptide growth factor, heregulin, leads to direct and rapid phosphorylation of ER on tyrosine residues. This is followed by interaction between ER and the estrogen-response elements in the nucleus and production of an estrogen-induced protein, progesterone receptor. In addition, overexpression of HER-2 receptor in estrogen-dependent tumor cells promotes ligand-independent down-regulation of ER and a delayed autoregulatory suppression of ER transcripts. These data demonstrate a direct link between these two receptor pathways and suggest one mechanism for development of endocrine resistance in human breast cancers.

Keywords: HER-2/neu ; estrogen receptor; heregulin; tyrosine phosphorylation; breast cancer

Introduction

Estrogens and peptide growth factors control the proliferation of breast cells. Alterations in the receptors for these agonists occur in human cancers in nature and lead to disruption of growth regulation (Harris et al., 1992). Among growth factor receptors, the most frequently implicated in human cancers have been members of the class I receptor tyrosine kinase family (erb B). Erb B tyrosine kinase receptors are overexpressed in two-thirds of human breast cancers and are associated with malignant transformation (Slamon et al., 1987; Slamon et al., 1989; Harris et al., 1992; Dougall et al., 1994). These receptors include the HER-2 (erb B2) and HER-3 (erb B3) proteins

which, together, constitute a high affinity functional receptor for heregulin (HRG), a ligand implicated in the autocrine/paracrine growth of breast epithelial cells (Carraway & Cantley, 1994; Sliwkowski et al., 1994). Receptors for estrogen are part of a family of steroid hormone receptors related to the viral erb A gene (Green & Chambon, 1988), and like the erb B proteins these receptors may play important pathogenic roles in breast cancer. Cross-coupling between erb B and estrogen receptor (ER) signal pathways in rodent uterine tissues has been reported (Ignar-Trowbridge et al., 1992) and is reminiscent of the cooperativity between viral erb A and erb B oncogenes in the malignant transformation of avian hematopoietic cells (Beug & Graf, 1989). Direct interaction between erb B signal pathways and ER in human breast cancer cells is the subject of the current studies.

Upon estradiol binding, ER interacts with specific estrogen-response elements (ERE) in the vicinity of target genes and modulates their transcription (Green & Chambon, 1988; Smith et al., 1993). The HER-2 receptor, with intrinsic tyrosine kinase activity, is believed to promote signal transduction along specific phosphorylation cascades (Harris et al., 1992; Silvennoinen et al., 1993; Dougall et al., 1994), with recruitment of proteins that serve as a link in activation of ras, inositol triphosphate, and, possibly, other signaling pathways to the nucleus (Silvennoinen et al., 1993). Phosphorylation of ER on tyrosine and/or serine residues has been associated with functional changes in both hormone binding and nuclear localization (Arnold et al., 1994; Kuiper & Brinkmann, 1994; Le Goff et al., 1994) and may be a link to kinase-mediated growth factor pathways. Blockade of estrogen-induced growth of breast tumor cells by tyrosine kinase inhibitors provides further evidence of the importance of tyrosine kinase pathways in estrogen action (Reddy et al., 1992).

Expression of either HER-2 or ER in human breast cancer provides important prognostic information (Slamon et al., 1987; Slamon et al., 1989b; Nicholson et al., 1990; Benz et al., 1992; Wright et al., 1992; Borg et al., 1994; Elledge et al., 1994). There are considerable data showing an association between HER-2 overexpression and the ER-negative phenotype (Zeilinger et al., 1989; Adnane et al., 1989), and failure of antiestrogen therapy in patients with breast cancer correlates with erb B receptor expression (Nicholson et al., 1990; Wright et al., 1992). In view of the above data, a greater understanding of the possible influence of erb B genes on the estrogen response is needed. Although ER is known to modulate HER-2 gene expression (Read et al., 1990; Russell & Hung, 1992), we postulate that reciprocal regulation of ER by erb B pathways may also

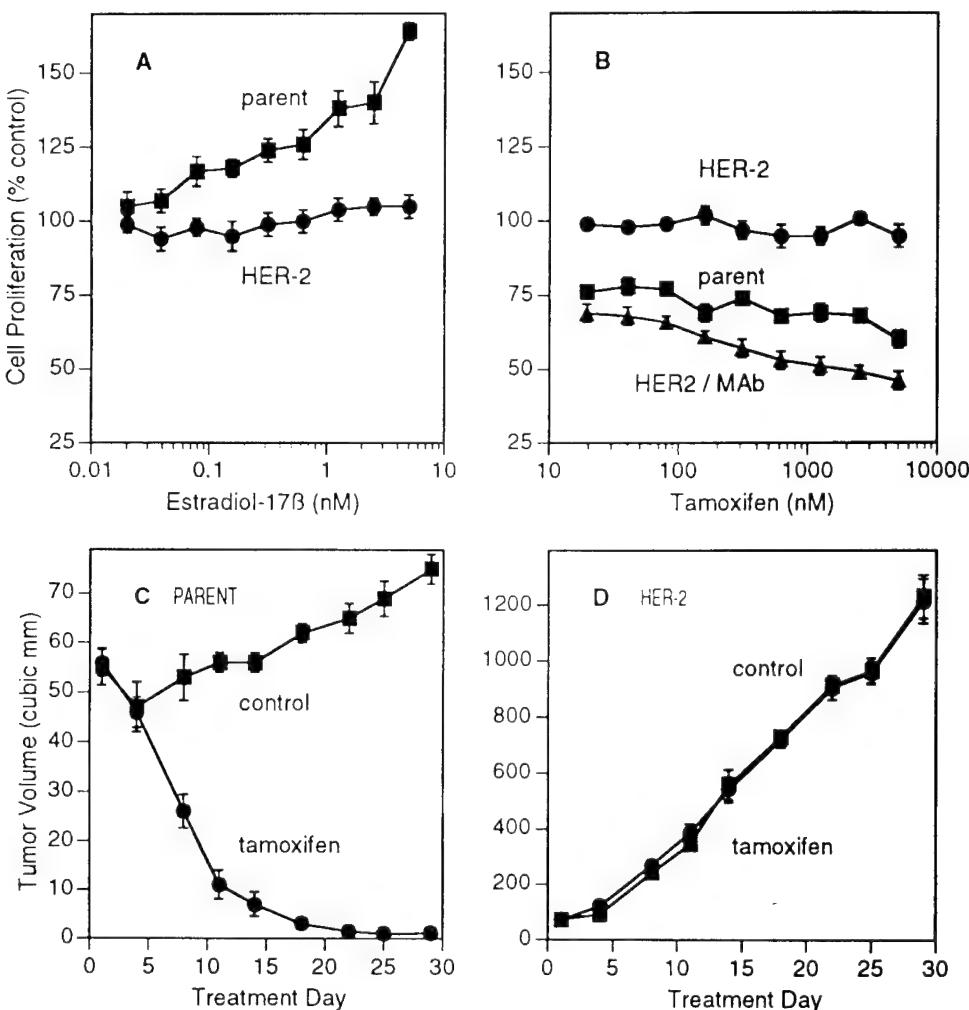


Figure 1 Overexpression of HER-2 gene in MCF-7 cells elicits resistance to endocrine therapy in vitro and in vivo. **(A)** Aliquots of 4×10^4 MCF-7 parental control or MCF-7 HER-2 cells were plated in 96-well plates in vitro. For experiments with estradiol-17 β , cells were plated in estrogen-free media. Following cell adherence, media supplemented with estradiol-17 α or vehicle control were added. After incubation at 37°C for 72h, plates were washed and stained with crystal violet, with intensity of staining correlating with cell growth. We found no response to 5nM estradiol-17 α in either cell line, confirming hormone specificity of the response (data not shown). **(B)** For experiments with tamoxifen, cells were plated in vitro in media with 5% serum. Results are given as % control cell proliferation for each group. Three different clones of MCF-7 HER-2 cells with 2-5 copies of HER-2 gene per cell, as well as a pool of MCF-7 HER-2 cells obtained by fluorescence-activated cell sorting, exhibited comparable levels of insensitivity to tamoxifen (data not shown). No significant difference in cellular accumulation of [3 H]-4OH-tamoxifen by MCF-7 parent as compared to MCF-7 HER-2 cells was found in three experiments (data not shown). **(C)** Antiestrogen sensitivity of MCF-7 cells without HER-2 overexpression in vivo. MCF-7 cells were inoculated sc in ovariectomized, athymic mice which were primed with estrogen. After 10 days, animals with tumors of comparable size were randomized to treatment with tamoxifen (5 mg sustained-release pellet /mouse s.c.) or control vehicle for 28 days. Tumor volumes of MCF-7 parental control cells with and without antiestrogen therapy were recorded. **(D)** Antiestrogen sensitivity of MCF-7 HER-2 cells in vivo. Cells with HER-2 overexpression were inoculated sc in ovariectomized, athymic mice which were primed with estrogen. After 10 days, animals with tumors of comparable size were randomized to therapy with tamoxifen as above.

occur, fostering hormone-independent growth in breast cancer.

To evaluate this hypothesis, we utilized estrogen-responsive, human breast cancer cells with defined levels of ER and derived from a common parental lineage to develop transfectants which are identical to their parental counterparts except for the expression of either the HER-2 or heregulin genes. These cells were then evaluated with regard to ER expression and estradiol binding as well as their response to estrogens, antiestrogens and the development of an estrogen-independent phenotype. The results of these studies give insight into how molecular

alterations leading to excess production of HER-2 receptors or heregulins may disrupt hormonal control and lead, in turn, to subversion of ER pathways for promotion of malignant growth.

Results

Overexpression of the HER-2 Receptor Promotes Estrogen-Independent Growth of Human Breast Cancer Cells

In view of substantial clinical evidence showing an inverse relation between the expression of HER-2 and ER (Adnane

et al., 1989; Zeillinger et al., 1989), we investigated the potential effect of HER-2 overexpression on ER expression and the genesis of estrogen-independent growth. This was accomplished using MCF-7 breast cancer cells which have single copies of HER-2 gene, no expression of HRG and require estrogen for growth (Read et al., 1990; Holmes et al., 1992). Transfection of a full length HER-2 cDNA into MCF-7 cells using a retroviral vector results in the introduction of 2-5 copies of the gene /cell and overexpression of HER-2 receptor (MCF-7 HER-2; Chazin et al., 1992). As shown in Fig. 1A, estradiol promotes a dose-dependent increase in proliferation of MCF-7 control cells *in vitro* ($P < 0.001$), but hormone doses of up to 5 nM elicit no significant effect on growth of MCF-7 HER-2 cells. As expected, treatment of MCF-7 parental cells with tamoxifen results in a significant reduction in cell proliferation ($P < 0.01$; Fig. 1B). However, MCF-7 HER-2 cells are unaffected by tamoxifen. These data indicate that overexpression of HER-2 gene in MCF-7 cells promotes insensitivity to both estradiol and tamoxifen *in vitro*.

Antiestrogen sensitivity of MCF-7 breast cancer cells was also tested *in vivo* using ovariectomized, athymic mice primed with estrogen for 10 days. Confirming prior studies (cf. Vignon et al., 1987; Wakeling, 1993), growth of MCF-7 control cells is markedly inhibited by tamoxifen (Fig. 1C) while MCF-7 HER-2 cells derived from these control cells exhibit resistance to tamoxifen treatment (Fig. 1D). Thus, overexpression of HER-2 receptors in human breast cancers growing *in vivo* is associated with failure of tamoxifen therapy and is consistent with what is seen clinically, i.e. HER-2-overexpressing tumors are resistant to tamoxifen.

To further confirm a link between HER-2 expression and hormone response, we tested the effects of an antibody to HER-2 on hormone response. If HER-2 overexpression plays a direct role in hormone resistance, then down-regulation of HER-2 receptor may result in a reversion to a more hormone-responsive phenotype. Monoclonal antibody rhuMAb HER-2 is a humanized form of the murine 4D5 antibody which is directed to the external domain of HER-2 and inhibits growth of cells with HER-2 overexpression (Carter et al., 1992). The antibody, a partial or weak agonist to the HER-2 receptor, promotes its down-regulation and blocks cell proliferation (Shepard et al., 1991). MCF-7 HER-2 cells were used to evaluate the effect of the antibody on the response of HER-2-overexpressing cells to tamoxifen therapy *in vitro*. As expected, rhuMAb HER-2 reduces breast cell proliferation to $88 \pm 3\%$ of controls when given alone (data not shown), but, more importantly, a further suppression of cell growth occurs with treatment with the antireceptor antibody and tamoxifen, indicating a return to hormone responsiveness (Fig. 1B). The latter effect is not significantly different from the response to tamoxifen alone in MCF-7 parent cells (Fig. 1B). These studies further support the involvement of the HER-2 receptor in mediation of hormone sensitivity.

Heregulin Activates the HER-2 Receptor and Stimulates Breast Cancer Cell Growth in the Absence of Estrogen

Heregulin- β 1 is a recombinant peptide with an EGF-like domain (Holmes et al., 1992) which activates the HER-2 receptor protein, a transmembrane tyrosine kinase. The recombinant HRG exhibits high-affinity binding to HER-2 / HER-3 heterodimers resulting in phosphorylation of HER-2 and enhanced proliferation of breast tumor cells *in vitro* (Holmes et al., 1992; Sliwkowski et al., 1994). To confirm

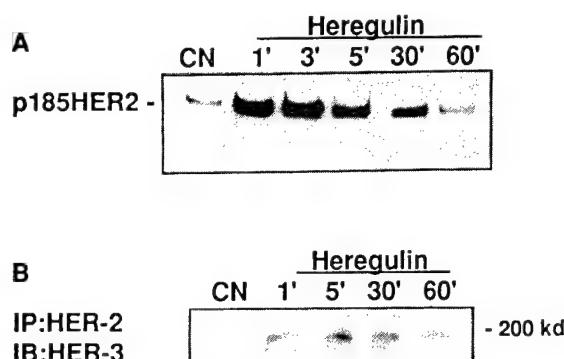


Figure 2 Heregulin promotes time-dependent HER-2 receptor phosphorylation and enhances HER-2 and HER-3 receptor association in MCF-7 HER-2 cells. (A) Effect of HRG treatment on HER-2 receptor phosphorylation. MCF-7 HER-2 cells were treated *in vitro* with HRG at 10 nM for 1 to 60 minutes. Lysates were prepared and processed as described in Materials and Methods. Samples were immunoprecipitated with anti-phosphotyrosine antibody before electrophoresis and Western blotting with anti-HER-2 antibody. HER-2 normally occurs as a 185 kd protein (Slamon et al., 1987). (B) Effect of heregulin on association of HER-2 and HER-3 receptors. MCF-7 HER-2 cells were treated *in vitro* with HRG at 10 nM for 1 to 60 minutes. Lysates were prepared and processed as above. Samples were immunoprecipitated with anti-HER-2 antibody (IP:HER-2; Slamon et al., 1987; Slamon et al., 1989a) prior to electrophoresis and Western blotting with anti-HER-3 antibody (IB:HER-3). HER-3 normally occurs as a 180 kd protein (Sliwkowski et al., 1994)

the activation of HER-2 with heregulin treatment in our system, we assessed *in vitro* tyrosine phosphorylation of the MCF-7 HER-2 cells in response to HRG. After HRG administration, these cells show a marked time-dependent increase in HER-2 tyrosine phosphorylation which becomes evident after one minute and peaks within thirty minutes (Fig. 2A). In addition, treatment with HRG in MCF-7 HER-2 cells promotes the enhanced association of HER-2 and HER-3 receptors, which becomes evident within one minute after HRG stimulation (Fig. 2B). These observations are consistent with earlier reports showing that HER-2 / HER-3 receptor heterodimers comprise a high-affinity receptor for HRG (Sliwkowski et al., 1994).

Due to its activating effect on the HER-2 kinase, activity of HRG on hormone-dependent growth of MCF-7 breast cells was evaluated (Fig. 3A). MCF-7 cells were implanted in ovariectomized mice without estrogen and treated with HRG or estradiol for 3 wks. Estrogen-dependent MCF-7 parent cells fail to grow in ovariectomized mice in the absence of estrogen, and, as expected, estradiol promotes an increase in growth of MCF-7 tumor nodules ($P < 0.001$; Fig. 3A). HRG treatment can also maintain the growth of these cells in ovariectomized mice even in the absence of estrogen ($P < 0.001$). These results suggest either that HRG stimulates an alternate growth pathway in the absence of hormone or that HRG itself may be an estrogen-related growth factor that directly acts in estrogen-dependent growth of breast cancer.

To further assess the effects of HRG on hormone-dependent growth, MCF-7 cells were tested after stable transfection with a full-length HRG- β 1 cDNA (Holmes et al., 1992). MCF-7 control cells do not produce HRG, but the transfected MCF-7 cells, designated MCF-7 HRG, show substantial levels of HRG expression, with cellular

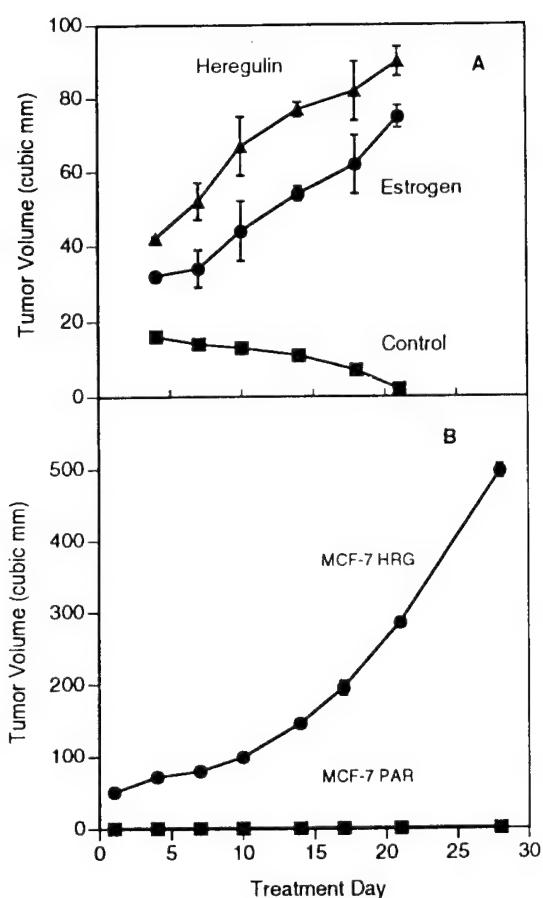


Figure 3 Heregulin stimulates breast tumor cell growth in the absence of estrogen. (A) Effect of HRG or estradiol-17 β (E β) on growth of MCF-7 parental cells in ovariectomized, athymic mice. At time of cell inoculation sc (5×10^7 cells/mouse), treatment with heregulin at 2 mg/kg or vehicle control (Control) was started sc and continued every other day for 3 wks. For comparison, athymic /ovariectomized mice were also treated with sc estrogen pellets beginning at the time of cell inoculation. (B) Tumorigenic potential of MCF-7 control (PAR) and MCF-7 HRG cells in ovariectomized nude mice with no estrogen supplementation. Protocols are described in Materials and Methods. Similar estrogen-independent growth was found in other clones of MCF-7 cells with expression of HRG (data not shown)

secretion of HRG averaging 4 ng / ml culture medium / day (data not shown). We tested the tumorigenic potential of the MCF-7 HRG cells in ovariectomized nude mice with no estrogen supplementation. MCF-7 cells which do not secrete HRG will not grow in athymic, ovariectomized mice without estrogen, while HRG-producing MCF-7 cells are tumorigenic in the absence of estrogen (Fig. 3B).

Activation of the HER-2 Receptor Elicits Down-Regulation of Estrogen Receptor Binding and Estrogen Receptor Transcripts

It is known that treatment of human breast cells with estrogen elicits a down-regulation of ER (Read et al., 1989; Ree et al., 1989; Borras et al., 1994). In the current study, the effects of overexpression or activation of HER-2 on specific binding of [3 H]-estradiol were tested in MCF-7 cells. Parental cells bind estradiol with both high affinity and capacity. However, MCF-7 cells with overexpression of the HER-2 gene demonstrate a significant reduction in

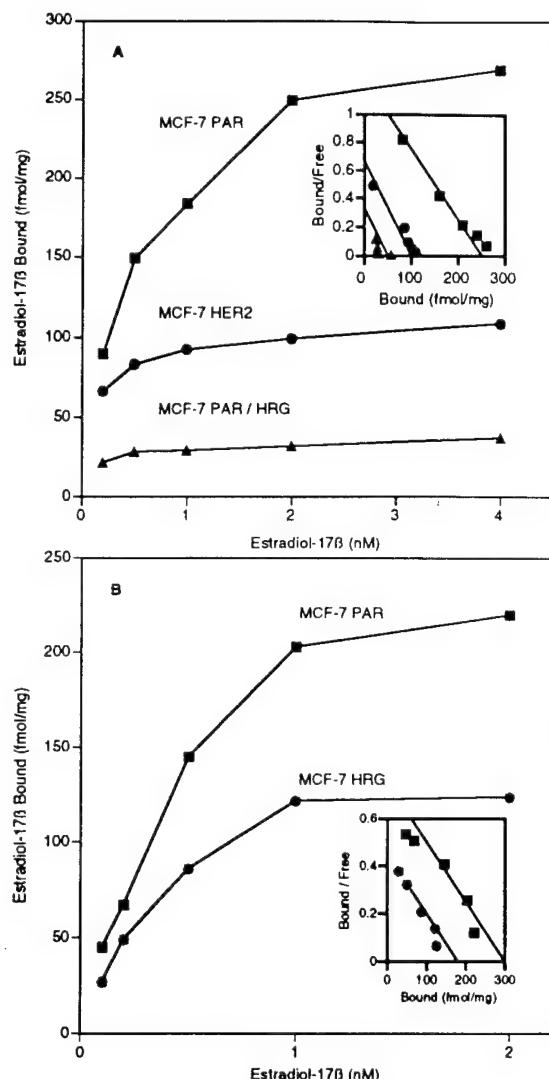


Figure 4 Activation of HER-2 receptor elicits down-regulation of estrogen receptor binding capacity in MCF-7 breast cancer cells. (A) Effects of overexpression or activation of HER-2 on specific binding of [3 H]-estradiol to MCF-7 control (MCF-7 PAR) and MCF-7 HER-2 cells. Binding in parental cells treated in vitro with 10 nM HRG for 72 hours is also shown (MCF-7 PAR/HRG). Scatchard analyses of the binding data to determine estrogen-binding capacity (B_{max}) and the affinity of hormone binding (K_d) are shown in the inset using the same symbols as in the main graph. Values for the K_d of estradiol binding to MCF-7 PAR, HER2 and PAR/HRG cells were 2.5×10^{-10} M, 1×10^{-10} M, and 1.3×10^{-10} M, respectively. Estradiol binding capacity in MCF-7 PAR, HER2 and PAR/HRG cells was 275 fmol/mg, 105 fmol/mg and 35 fmol/mg, respectively (see inset). (B) Specific binding of [3 H]-estradiol to MCF-7 control (MCF-7 PAR) and MCF-7 HRG cells. Scatchard analyses of control and HRG-producing MCF-7 cells were done to evaluate estradiol binding capacity and binding affinity (see inset). Values for the K_d of estradiol binding to MCF-7 PAR and MCF-7 HRG cells were 4.6×10^{-10} M, and 3.5×10^{-10} M, respectively. Estradiol binding capacity in MCF-7 PAR and MCF-7 HRG cells was 298 fmol/mg and 158 fmol/mg, respectively (see inset)

estrogen-binding capacity (B_{max}) with no change in affinity of hormone binding (K_d ; Fig. 4A). In addition, treatment of MCF-7 parental cells with HRG elicits a similar decrease in estrogen-binding capacity. Binding capacity for estrogen was further tested in MCF-7 control

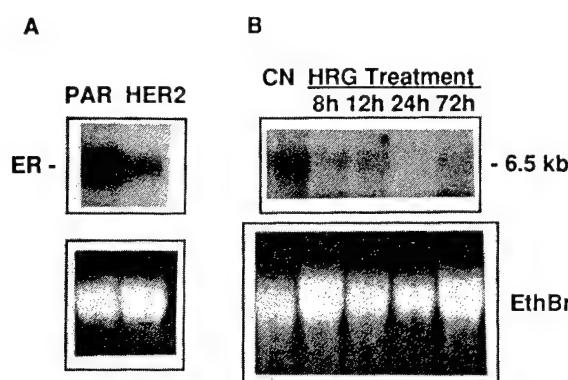


Figure 5 Activation or overexpression of HER-2 receptor elicits down-regulation of estrogen receptor transcripts. (A) Analyses of RNA from MCF-7 control (PAR) and MCF-7 HER-2 cells. Cell RNA was isolated 7 days after plating cells in estrogen-free media and subjected to Northern blot analysis (Chazin et al., 1992; Slamon et al., 1987), with hybridization of the resulting blot with human ER cDNA. A major ER mRNA transcript of approximately 6.5 kilobases has been described before. The ethidium bromide staining pattern of matched ribosomal RNA is shown for comparison. (B) Heregulin administration in vivo down-regulates ER transcripts in MCF-7 HER-2 cells grown as xenografts in athymic, ovariectomized mice over 72 hours. At the time of cell inoculation sc (5×10^7 cells/mouse), treatment with HRG at 2 mg/kg was started sc and continued every other day. Paired mice were given vehicle control (CON) as shown at time zero for comparison. The ethidium bromide staining pattern of matched ribosomal RNA is shown for comparison.

and MCF-7 HRG cells (Figure 4B), and demonstrate that HRG-producing MCF-7 cells exhibit a specific estradiol binding capacity half that of control cells. Thus, either overexpression or activation of HER-2 elicits a down-regulation in ER similar to that found after treatment with estrogen.

It is also known that treatment of MCF-7 cells with estrogen leads to a pronounced down-regulation of ER transcripts. This process is believed to be mediated by an active ER and considered to be part of an autoregulatory circuit limiting the duration of estrogen action (Read et al., 1989; Ree et al., 1989; Borras et al., 1994). To test this phenomenon in our system, analyses of RNA from MCF-7 control and MCF-7 HER-2 cells was performed. These studies show that the major ER transcript of 6.5 kb is reduced in breast cells that overexpress HER-2 gene compared to controls (Fig. 5A), and that treatment of MCF-7 HER-2 cells in vivo with HRG elicits a similar decrease in ER transcripts over 72 hours (Fig. 5B).

Activation of the HER-2 Receptor Leads to Tyrosine Phosphorylation and Enhanced Nuclear Binding of the Estrogen Receptor

As noted above, phosphorylation of ER on tyrosine (Koffman et al., 1991; Migliaccio et al., 1991; Castoria et al., 1993) and serine (Arnold et al., 1994; Le Goff et al., 1994) residues has been associated with functional changes in hormone binding and nuclear localization and may represent a link to tyrosine kinase-mediated growth factor pathways. To test if ER is a substrate for phosphorylation by a tyrosine kinase receptor activated by HRG, we treated MCF-7 cells with HRG. MCF-7 control cells (which

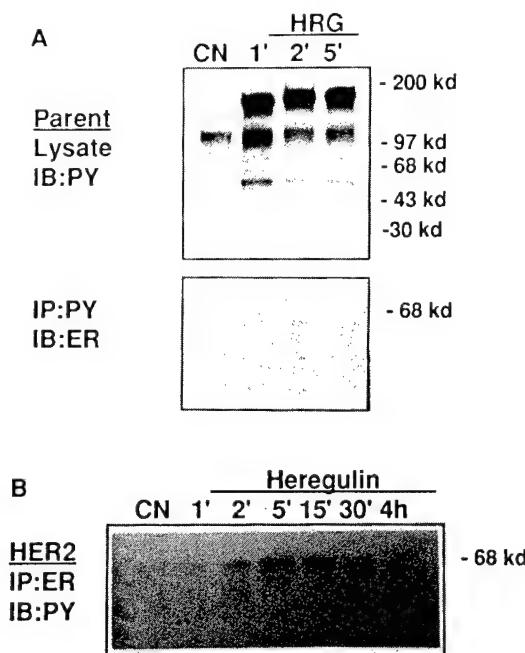


Figure 6 Activation of HER-2 receptor leads to tyrosine phosphorylation of estrogen receptor. (A) MCF-7 parent cells were treated in vitro with 10 nM HRG or control vehicle (CN) for 1 to 5 minutes. Lysates were prepared and processed as described in Materials and Methods. In the upper panel, a sample of the total lysate was analysed by electrophoresis and then evaluated by immunoblot with antiphosphotyrosine antibody (IB:PY). In the lower panel, the lysate was first immunoprecipitated with antiphosphotyrosine antibody (IP:PY) before electrophoresis and Western blotting with anti-ER antibody (IB:ER). Human MCF-7 cell ER normally occurs as a 65- to 70-kd protein (Horigome et al., 1987). (B) MCF-7 HER-2 cells were treated in vitro with 10 nM HRG or control vehicle (CN) in the absence of estrogen to evaluate tyrosine phosphorylation of ER from 1 min to 4 hours using methods described in Materials and Methods. The total lysate was treated first by immunoprecipitation with anti-ER monoclonal antibody (IP:ER; not shown), followed by electrophoresis and immunoblotting with antiphosphotyrosine antibody (IB:PY)

express normal amounts of the HER-2 receptor) treated with HRG in the absence of estrogen show a prominent increase in tyrosine phosphorylation of several cell proteins, especially at 185 kd, and demonstrate a marked time-dependent tyrosine phosphorylation of ER protein (Fig. 6A). Phosphorylation can be seen as early as 1 to 2 min after HRG treatment. In MCF-7 HER-2 cells, HRG promotes a similar acute increase in tyrosine phosphorylation of ER, with maximal phosphorylation occurring at 5-15 min and declining by 30 min (Fig. 6B). These results demonstrate a direct link between the HER-2/HRG pathway and ER tyrosine phosphorylation and are consistent with recent studies showing ligand-independent activation of steroid hormone receptors suggesting that molecular activation of ER may not depend exclusively on estrogen binding (Nelson et al., 1991; Ignar-Trowbridge et al., 1992; Smith et al., 1993).

To extend these observations to biologic phenomenon known to be associated with ER phosphorylation / activation, we performed a series of subcellular fractionation experiments in cells treated with HRG in the absence of estradiol. Published studies have shown that estrogen treatment rapidly enhances affinity of ER for

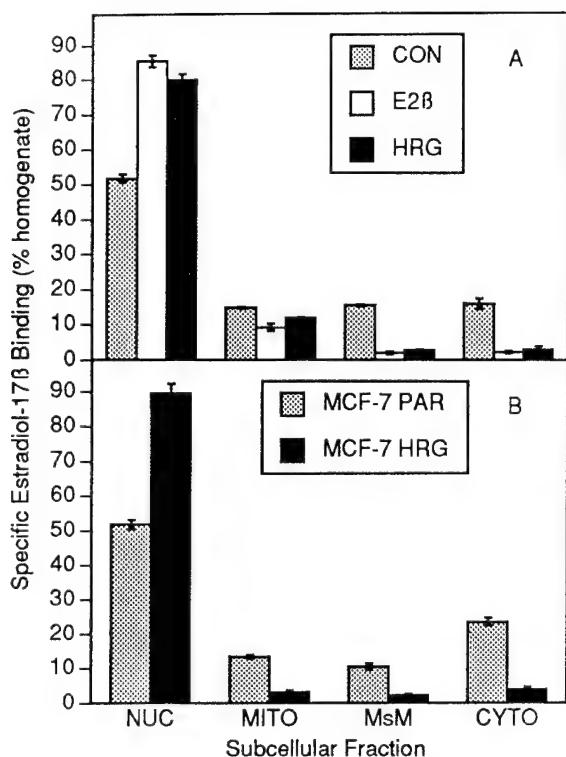


Figure 7 Activation of HER-2 receptor leads to enhanced nuclear binding and retention of estrogen receptor. (A) Subcellular fractionation studies in cells treated with HRG in the absence of estradiol. MCF-7 cells after treatment with 1 nM estradiol-17 β , 10 nM hereregulin, or control vehicle for 1 hour were homogenized under controlled conditions and subcellular fractions were purified by established methods. Specific binding of [3 H]estradiol was quantitated in nuclear (NUC), mitochondria-lysosomal (MITO), microsomal (MsM) and cytosolic (CYTO) fractions. Specific binding of [3 H]estradiol in cells treated with estradiol-17 β (E2 β) was determined by a ligand-exchange assay at 37° C, while that in the presence of hereregulin (HRG) or control vehicle (CON) was assessed by standard methods (Pietras & Szego, 1979; Horigome et al., 1987; Pietras & Szego, 1984). Using a monoclonal antibody to ER, we also find that HRG treatment promotes enhanced immunohistochemical detection of ER in nuclear fractions of disrupted cells as well as in nuclei of intact cells (data not shown). Distribution of cell protein in the several cell fractions of MCF-7 parent cells treated with estradiol-17 β , hereregulin or control was not significantly different among the three treatment groups. Under all treatment conditions, DNA recovery was greater than 91% of homogenate levels in nuclear fractions, and the specific activity of 5'-nucleotidase, a plasma membrane marker enzyme, was enriched predominantly in microsomal membrane fractions (more than 38% of homogenate in all treatments; data not shown). These results are similar to those reported by others (Pietras & Szego, 1984; Welshons et al., 1993) and confirm the integrity of our subcellular fractions. (B) Specific binding of [3 H]estradiol was quantitated in subcellular fractions of MCF-7 control (MCF-7 PAR) and HRG-producing MCF-7 (MCF-7 HRG) cells to assess the influence of long-term HRG exposure on distribution of ER. Specific binding of [3 H]estradiol was quantitated in nuclear (NUC), mitochondria-lysosomal (MITO), microsomal (MsM) and cytosolic (CYTO) fractions as above. Distribution of cell protein was determined in fractions of control and HRG-producing MCF-7 cells and was not significantly different among the treatment groups.

chromatin, leading to its retention in the nuclear fraction after cell homogenization (Pietras & Szego, 1984; Green & Chambon, 1988; Welshons et al., 1993). In the current study, this phenomenon was quantitated in MCF-7 cells

after treatment with estradiol-17 β or hereregulin. Cells were homogenized under controlled conditions and subcellular fractions were purified by established methods (Pietras & Szego, 1979; Pietras & Szego, 1984). Specific binding of [3 H]estradiol, a measure of ER binding activity, was then quantitated in subcellular fractions including a nuclear, microsomal, mitochondria-lysosomal and cytosolic fraction (Pietras & Szego, 1979). Treatment with estradiol-17 β elicited the anticipated increased specific binding of [3 H]estradiol in the nuclear fraction as compared to controls (Figure 7A). Like estradiol, hereregulin promoted a significant increase in nuclear binding of [3 H]estradiol despite the fact that it was used in the absence of the steroid hormone (Figure 7A). Finally, specific binding of [3 H]estradiol was also quantitated in subcellular fractions of MCF-7 control and MCF-7 HRG cells to assess the influence of long-term HRG exposure on distribution of ER. Nuclear localization of [3 H]estradiol binding in MCF-7 HRG cells was almost twice that found in nuclear fractions of control cells (Figure 7B). These data indicate that either exogenous treatment with or endogenous production of HRG in MCF-7 cells facilitates ER binding in the nucleus.

Activation of the HER-2 Receptor Promotes Estrogen-Independent Nuclear Signaling by the Estrogen-Responsive Element (ERE) and Production of an Estrogen-Induced Protein

Since interaction of ER with nuclear ERE is prerequisite for activation of ER-induced transcription (Green & Chambon, 1988), regulation of ERE by HRG was tested in MCF-7 cells transiently transfected with a reporter plasmid containing an ERE upstream of a chloramphenicol acetyltransferase (CAT) gene. As shown in Figure 8A, estradiol-17 β activates the ERE-CAT reporter construct in MCF-7 cells. In the absence of estrogen, however, HRG also activates the ERE-CAT gene, and this effect can be abolished by preincubation with the pure antiestrogen, ICI 182,780, which works at the level of the ER-ERE interaction (Parker, 1993). These data demonstrate that HRG can promote estrogen-independent nuclear signaling by ER.

The ERE-CAT gene was also transiently transfected into MCF-7 cells with or without HER-2 gene overexpression. In the absence of estrogen, MCF-7 HER-2 cells show enhanced ERE-CAT gene activity as compared to that of MCF-7 control cells (Figure 8A). The increase in ERE-CAT activity associated with HER-2 overexpression can be supplemented further by treatment with estradiol-17 β ($P<0.01$). The effect associated with HER-2 overexpression can also be significantly reduced by incubation with the pure antiestrogen, ICI 182,780 ($P<0.01$). To assess the influence of endogenous HRG synthesis, the ERE-CAT gene construct was transiently transfected in MCF-7 cells with or without HRG gene expression. As shown in Figure 8B, MCF-7 HRG cells also show increased ERE-CAT activity under basal conditions as compared to MCF-7 parental cells ($P<0.001$). The activity in MCF-7 HRG cells is enhanced further by treatment with estradiol-17 β ($P<0.05$), but is not increased by incubation with exogenous HRG (Figure 8B). Thus, activation of the HER-2 receptor by HRG or overexpression of the HER-2 receptor contributes to regulation of ER transcriptional activity in the absence of estrogen.

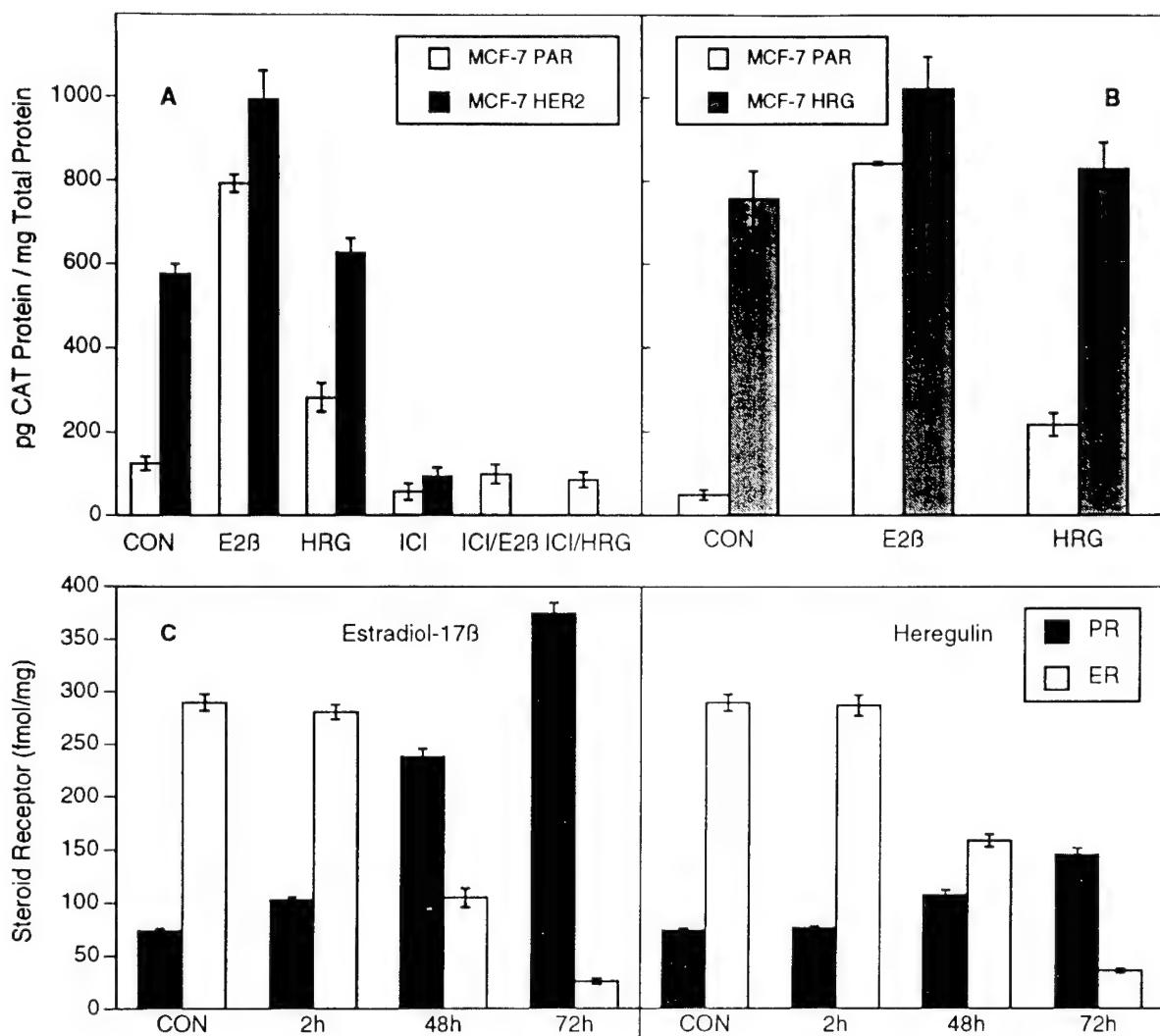


Figure 8 Activation of HER-2 receptor promotes estrogen-independent nuclear signaling by estrogen-responsive element (ERE) and production of estrogen-induced protein. (A) Heregulin promotes activation of ERE-CAT reporter gene after transient transfection in MCF-7 cells without (MCF-7 PAR) or with (MCF-7 HER2) overexpression of HER-2 gene. We have used a reporter plasmid containing a palindromic ERE, derived from the vitellogenin A2 promoter, and the CAT gene driven by a partial promoter sequence of thymidine kinase (Ernst et al., 1991; Lees et al., 1989). Substituting the basic reporter plasmid pBLCAT2 for pERE-BLCAT offers an additional control for specificity of the DNA-binding site in the regulatory sequence of the reporter gene (ERE). MCF-7 cells were used to establish transient transfection assays that allow the determination of ERE-dependent induction of CAT activity. CAT protein was assessed by established methods (De Maio & Buchman, 1990). Activity of control (CON), 1 nM estradiol-17 β (E2 β) and 10 nM heregulin (HRG) for 24h was assessed using these transfected cells with or without ERE. The specificity of this ligand-dependent CAT activation was verified by treatment of cells with the biologically inactive estradiol stereoisomer, estradiol-17 α , and the non-estrogenic steroid, progesterone. At concentrations of 2 nM, both compounds have no significant effect on induction of CAT activity as compared to controls (data not shown). In addition, neither E2 β nor HRG elicited any change in the basal level of activity of the CON-CAT gene construct in MCF-7 parent cells. Further, both agents were ineffective in regulating the activity of ERE-CAT gene transiently transfected in HBL-100 breast cells which have no detectable level of endogenous ER (ER<5 fmol/mg protein; data not shown). The effect of preincubation with the pure antiestrogen, ICI 182,780 (ICI; 10 nM), on unstimulated MCF-7 HER2 cells and on the effects of estrogen and HRG in MCF-7 PAR cells was also tested. (B) Effect of 1 nM estradiol-17 β (E2 β) and 10 nM heregulin (HRG) on ERE-CAT reporter gene after transient transfection in MCF-7 cells without (MCF-7 PAR) or with (MCF-7 HRG) heregulin gene. Methods are described in Materials and Methods. (C) Timecourse of changes in estrogen receptor (ER) and progesterone receptor (PR), an estrogen-induced protein, in MCF-7 cells treated in vitro with 1 nM estradiol-17 β (left panel), 10 nM HRG (right panel) for 2h to 72h as compared to control solution treatments (CON). Specific steroid binding was determined using whole cell binding assays, and Scatchard analyses of the binding data were utilized to obtain quantitative estimates of the number of steroid binding sites/cell as before (Pietras & Szego, 1979). The results are shown as fmol specific hormone bound per mg cell protein

To further assess potential overlapping modes of action between HRG and ER pathways, the time course of changes in ER and progesterone receptor (PR), an estrogen-induced protein, were evaluated in MCF-7 cells treated in vitro with estrogen or HRG. Treatment of MCF-7 cells with estradiol-17 β for 2h to 72h results in a progressive induction of PR levels to more than 5-fold over control levels ($P<0.001$; Fig.

8C). Concurrently, estrogen elicits a complementary autoreduction in ER content to about 12% of that found in control cells ($P<0.001$). Treatment of MCF-7 cells with HRG over the same timecourse elicits a similar ER and PR phenotype in that PR binding capacity increases to twice that of control cells ($P<0.001$), while ER content falls to about 20 % of that found in control cells (Fig. 8C). Thus,

HRG, like estradiol, promotes a reduction in ER and an induction of PR in breast tumor cells. It is important to note, however, that, with continuous exposure of MCF-7 cells to HRG for 120 hours, PR content is reduced to 78 % of control levels while ER content remains at 18 % of controls (both at $P < 0.05$; data not shown). These data demonstrate that prolonged exposure to HRG can generate a steroid hormone receptor-negative (ER- / PR-) phenotype.

Discussion

There is considerable evidence that growth of human breast epithelial cells is regulated by receptors for both steroid hormones and peptide growth factors (Harris et al., 1992). Alterations in members of both of these classes of receptors have been associated with various aspects of human breast cancer. Overexpression of the HER-2 receptor is found in approximately 25-30% of breast cancers and is associated with a poor prognosis (Slamon et al., 1987; Slamon et al., 1989a; Press et al., 1994). Most commonly, this overexpression is a result of amplification of the gene (Slamon et al., 1989a; Harris et al., 1992). Detectable ER is present in about 75% of breast cancers at diagnosis. Like HER-2 overexpression, alteration in ER expression is associated with a poor clinical outcome (Elledge et al., 1994). Recent data have shown a correlation between HER-2 overexpression and an ER-negative phenotype in breast cancer (Adnane et al., 1989; Press et al., 1994). Consistent with this observation is the fact that tumors containing the HER-2 alteration tend to be resistant to tamoxifen therapy (Nicholson et al., 1990; Benz et al., 1992; Borg et al., 1994). Taken together, the above data indicate that overexpression of the HER-2 gene may be related to the genesis of resistance to endocrine therapy in some breast cancers.

Several potential mechanisms of development of estrogen resistance in human breast cancer have been postulated (Osborne & Fuqua, 1994), but a major barrier to the study of progression from a responsive to a resistant phenotype is the lack of adequate model systems. To address this with regard to HER-2, we developed human breast cancer lines derived from a common lineage containing defined levels of ER but with differing levels of HER-2 expression. The parental MCF-7 cell line does not have the HER-2 alteration, i.e. it has a single copy of the gene per chromosome 17 and expresses normal levels of the gene product. The current studies show that introduction of additional copies of the gene with an attendant increase in expression of the receptor protein leads to a reduction in estrogen binding capacity, and that activation of the receptor with HRG elicits a further and sustained decrease in ER transcripts. Interestingly, a similar down-regulation of ER is found in rat mammary carcinomas induced by the mutant murine *neu* oncogene (Wang et al., 1992). These data demonstrate a reproducible connection between HER-2 overexpression or activation and loss of ER binding activity and gene expression. The mechanism(s) linking the two systems, however, are as yet incompletely defined. Is HER-2 overexpression or activation mimicking or in some way substituting for events which occur when estrogen interacts with its receptor, most notably down-regulation of the ER (Ree et al., 1989; Read et al., 1989; Borras et al., 1994)? It is known that upon binding the ER, estrogen induces phosphorylation of its

receptor (Kuiper & Brinkman, 1994). In the calf uterus, a tyrosine kinase has also been implicated to phosphorylate ER on tyrosine early in the course of hormone action (Castoria et al., 1993). In the human, there is some debate as to which amino acid residues are phosphorylated with evidence suggesting that serine (Denton et al., 1992; Le Goff et al., 1994; Arnold et al., 1994) and/or tyrosine (Koffman et al., 1991; Migliaccio et al., 1991; Castoria et al., 1993) residues are critical. After phosphorylation of ER, dimerization of the complex occurs leading to tight binding of ER to its specific estrogen-response elements (ERE) in DNA. Following ERE binding, the ligand/receptor complex functions as a nuclear transcription factor promoting the synthesis of specific proteins such as the progesterone receptor (Green & Chambon, 1988; Smith et al., 1993). Do any of the same phenomenon occur with regards to ER when the HER-2 receptor is activated? Our data demonstrate that activation of the HER-2 receptor in breast cancer cells by heregulin is associated with phosphorylation of ER on tyrosine residues. The effect is rapid and appears to parallel the enhanced nuclear retention of the receptor seen with estrogen. Moreover, HER-2 receptor activation leads to ligand-independent down-regulation of ER and expression of an estrogen-induced protein, the progesterone receptor (PR). These data clearly show convergence between the two ligand / receptor pathways and provide confirmation of previous findings which suggest that steroid receptor function in some tissues may be regulated, in part, by cross-talk from membrane receptor pathways (Nelson et al., 1991; Power et al., 1991; Ignar-Trowbridge et al., 1992; Ignar-Trowbridge et al., 1993; Smith et al., 1993). This hypothesis is supported further by the inhibitory effects of ICI 182,780 on ER-dependent protein induction in tumors with activation of HER-2 receptor pathways. These tumors are tamoxifen resistant, but ICI 182,780, a pure antiestrogen devoid of estrogenic activity, appears to function by a different mechanism than tamoxifen. Impaired dimerization of ER proteins in the presence of ICI 182,780 leads to alterations in turnover of the receptor proteins and interaction with the ERE (Parker, 1993). This molecular effect of the pure antiestrogen may interfere with ERE activation by HER-2 signal pathways in a similar fashion, i.e. inhibition of the interaction between the phosphorylated ER and the ERE. Independent studies on the genesis of resistance to endocrine therapy demonstrate that prolonged ligand-induced down-regulation of receptor results in a loss of hormone sensitivity that is reversible only if the ligand is withdrawn (Bellingham et al., 1992). In this way, long-term ligand-independent regulation of ER by a HER-2-mediated pathway may also lead to estrogen resistance. This suppression of ER would, in turn, lead to down-regulation of PR since the latter is induced by ER. Supporting this model is the observation that an ER-negative, PR-negative phenotype is, in fact, most commonly found in human breast cancers containing overexpression of HER-2 receptor (Adnane et al., 1989; Zeillinger et al., 1989; Press et al., 1994).

Other members of the class I tyrosine kinase receptor family may also affect ER pathways. EGF is reported to modulate cell growth and differentiation via ER in rat uterus (Nelson et al., 1991; Ignar-Trowbridge et al., 1992; Ignar-Trowbridge et al., 1993), while EGF and related peptides elicit some of their cellular actions by interaction



with ER in MCF-7 cells (Vignon et al., 1987; Read et al., 1989). Our findings demonstrate that heregulin supports growth of estrogen-dependent MCF-7 breast cells even in the absence of estrogen. Although independent work demonstrates that heregulins stimulate growth of breast cells with a complex of HER-2 and HER-3 receptors in vitro (Holmes et al., 1992; Carraway & Cantley, 1994; Sliwkowski et al., 1994), neu differentiation factor, which is structurally homologous with heregulin, has been reported to promote differentiation and growth inhibition rather than growth (Bacus et al., 1992). Heregulin is known to bind with high affinity to heterodimers of HER-2 and HER-3 (Carraway & Cantley, 1994; Sliwkowski et al., 1994), and likely with complexes including HER-4 protein (Plowman et al., 1993; Dougall et al., 1994). Conflicting findings on the growth effects of heregulins (Staebler et al., 1994) may be attributable, in part, to different preparations of ligands or to differences in the cellular complement of class I receptors making up functional heterodimers in a given cell. The hypothesis that another member of the HRG ligand family elicits different effects on ER-positive and ER-negative cells has also been proposed by other investigators (Kung et al., 1994). Our data clearly demonstrate that, in estrogen-dependent MCF-7 breast cancer cells, HRG promotes and maintains cell growth even in the absence of estrogen. The inverse correlation between ER and HER-2 receptors in invasive cancer has been poorly understood (Adnane et al., 1989; Press et al., 1994), but the present data suggest that estrogen resistance due to long-term suppression of ER by HER-2-mediated pathways may require alternative therapeutic approaches.

Materials and methods

Cell Lines

MCF-7 cells (American Type Culture Collection, Rockville, MD) were stably transfected with a vector containing the full-length cDNA of human HER-2 gene from a primary breast cancer tissue (Slamon et al., 1987; Slamon et al., 1989b). These cells are termed MCF-7 HER-2. The vector used for introduction of HER-2 gene into human cells contained full-length human HER-2 gene ligated into a replication-defective retroviral expression vector, pLXSN (Slamon et al., 1987; Slamon et al., 1989b; Chazin et al., 1992). The latter was done by ligating a 3.8 kb Nco I to Mst II fragment with the full HER-2 coding sequence but lacking a polyadenylation signal into an amphotrophic retroviral expression vector containing a Moloney murine leukemia virus promoter, a neomycin phosphotransferase gene and a packaging signal, but devoid of viral protein coding sequences; thus rendering the virus replication-defective. Virus-producing cells were prepared by a transient rescue procedure as described elsewhere (Chazin et al., 1992). The vector devoid of HER-2 but containing neomycin phosphotransferase gene for selection (with G418) was packaged in an identical fashion and served as a control to infect MCF-7 cells (MCF-7 Control). G418-resistant clones were selected from MCF-7 cells infected with retroviral vector with or without HER-2 cDNA and assayed for expression of HER-2 receptor (Chazin et al., 1992). MCF-7 HER-2 cell clones with 2-5 copies of HER-2 gene per cell were used in these studies. Alternatively, in some experiments, pools of retrovirus-infected MCF-7 cells were selected first for HER-2 receptor

overexpression by fluorescence-activated cell sorting using monoclonal anti-HER-2 receptor antibody 4D5 as described previously (Benz et al., 1992) and then screened further by subculture in the presence of G418.

Non-malignant human breast epithelial cells, HBL-100 (American Type Culture Collection), and all other cells were routinely plated in RPMI medium 1640 (GIBCO/BRL, Grand Island, NY) with 2mM glutamine and 1% penicillin G-streptomycin-fungizone solution (Irvine Scientific, Santa Ana, CA). Unless stated otherwise, medium with 10% heat-inactivated fetal bovine serum was used for standard plating conditions. In experiments requiring estrogen-free conditions, medium without phenol red and supplemented with 1% heat-inactivated, dextran-coated charcoal-treated fetal bovine serum was used for 72h prior to the start of the experiment. These are standard procedures for removing estrogens and steroid-like materials from culture media (Horigome et al., 1987; Welshons et al., 1993).

MCF-7 breast cancer cells with no endogenous production of HRG (American Type Culture Collection) were stably transfected with a vector containing full-length HRG- β 1 cDNA (Holmes et al., 1992). These cells are termed MCF-7 HRG. The vector used for introduction of HRG- β 1 gene into human cells contained full-length HRG- β 1 gene cloned into the expression vector pRK7 (Carter et al., 1992). This is a phagemid expression vector containing the human cytomegalovirus enhancer and promoter, a 5' intron and the simian virus 40 late polyadenylation signal. Expression vector devoid of HRG- β 1 cDNA was used as a control and was transfected into MCF-7 cells as described previously (Gorman et al., 1983). Production of HRG was assessed by competitive ligand-binding assay using [125 I]-HRG. With this assay, MCF-7 control cells showed no detectable level of HRG, while substantial levels were found in MCF-7 HRG cells (95% inhibition of ligand binding). A radioimmunoassay for HRG was also applied in selected experiments with use of a hamster monoclonal antibody to HRG (Holmes et al., 1992).

Heregulin- β 1 and Anti-HER-2 Antibodies

Heregulin- β 1 was prepared as described previously (Holmes et al., 1992; Sliwkowski et al., 1994). All preparations of recombinant HRG- β 1 were tested to verify high-affinity binding to HER-2/HER-3 heterodimers, and by phosphorylation of HER-2, as well as stimulation of proliferation of SKBR3 breast tumor cells (ATCC) in vitro (Holmes et al., 1992; Sliwkowski et al., 1994).

A humanized form of anti-HER-2 receptor monoclonal antibody 4D5 has been described before (Carter et al., 1992). The construct contains only the antigen-binding loops from murine 4D5 antibody in combination with human variable region framework residues plus IgG1 constant domains and is termed rhuMAb HER-2. Human IgG1 was used as control solution in appropriate experiments.

Quantitation of Cell Proliferation in Vitro

To assess proliferation of breast cells in vitro, aliquots of 4×10^4 cells were plated in 96-well microdilution plates. For experiments with estradiol-17 β , cells were initially plated in estrogen-free media (see above). Following cell adherence, media supplemented with estradiol-17 β , estradiol-17 α or

vehicle control was added. After incubation at 37°C for 72h, plates were washed and stained with crystal violet, with intensity of staining correlating with cell growth (Shepard et al., 1991; Chazin et al., 1992). For experiments with tamoxifen, cells were plated in media with 5% serum. Results are given as percent control of cell proliferation for each group.

Quantitation of Cell Growth in Athymic Mice in Vivo

MCF-7 cells (5×10^7 cells / mouse) were grown as subcutaneous (sc) xenografts in athymic, ovariectomized mice (Harlan Sprague-Dawley, Indianapolis, IN). In experiments with exogenous HRG administration, treatment with HRG at 2 mg / kg was started sc and continued every other day from the time of cell inoculation. Paired mice were given vehicle control for comparison. Therapy with estradiol-17 β (E β) was by implantation of estrogen pellets sc (1.7 mg/biodegradable carrier-binder pellet; Innovative Research of America, Toledo, OH) beginning at the time of cell inoculation.

In studies with antiestrogen agents in vivo, human breast cancer cells without (MCF-7 Control) and with (MCF-7 HER-2) HER-2 overexpression were inoculated into ovariectomized, athymic mice which had been primed with estrogen (1.7 mg / biodegradable pellet). After 10 days, animals with tumors of comparable size (50-100 mm³) were randomized to treatment with tamoxifen (5 mg sustained-release pellet /mouse sc; Innovative Research of America) or vehicle control for an additional 28 days. Tumor volumes of cells with or without antiestrogen therapy were then recorded. Five to six animals were included in each group, after randomization by body weight and tumor nodule size at the start of each experiment.

Tyrosine Phosphorylation of Estrogen Receptor and HER-2 Receptor

MCF-7 cells were examined for ER phosphorylation by Western blot using 4-15% SDS-polyacrylamide gradient gel electrophoresis methods (Slamon et al., 1987; Chazin et al., 1992). Cells were grown in 100 mm culture dishes to 80% confluence in estrogen-free media (RPMI 1640 media without phenol red and supplemented with 1% dextran-coated charcoal-treated FCS) and remained serum-free for 3h prior to addition of HRG (10nM) for various times at 37°C. Cells were rinsed with cold PBS three times and chilled at 0-4°C prior to lysis in 0.4 ml cold buffer containing 0.15 M NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 10 mM Tris (pH 7.4), 1 mM phenylmethylsulfonylfluoride, 0.7 μ g/ml pepstatin, 5 μ g/ml leupeptin, 10 μ g/ml aprotinin, 0.1 mg/ml soybean trypsin inhibitor, and 1 mM sodium orthovanadate (Sigma Chemical Company, St. Louis, MO) and then prepared otherwise as described before (Le Goff et al., 1994). Total protein concentration was determined by BCA assay (Pierce Biochemical, Arlington Heights, IL). Phosphorylation of ER was analyzed by immunoprecipitation with anti-ER monoclonal antibody H-222 (Abbott Laboratories, Chicago, IL). Immunoprecipitations were performed by incubating 0.25 mg lysate protein with 1 μ g/ml antibody overnight at 4°C with gentle agitation. Protein A/G immobilon (Pierce Biochemical) was added to precipitate the antigen-antibody complex. Immunoprecipitates were washed four times prior to electrophoresis of ER protein on a 4-15% SDS-polyacrylamide gradient gel (Horigome et al., 1987; Slamon

et al., 1987; Chazin et al., 1992). Proteins were then transferred to nitrocellulose and subjected to Western blot analysis using monoclonal 4G10 anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) for immunoblots and the ECL® (Amersham) detection method as described previously (Migliaccio et al., 1991; Chazin et al., 1992). In selected experiments, phosphorylation of ER or HER-2 receptor was analyzed by an alternate approach, using immunoprecipitation of 0.25 mg lysate protein with agarose-conjugated antiphosphotyrosine antibody (Upstate Biotechnology) according to the recommendations of the manufacturer, followed by immunoblotting with anti-ER or anti-HER-2 (Oncogene Science) monoclonal antibody. For electrophoresis of total lysate preparations, 25 μ g protein was used in appropriate experiments.

Co-immunoprecipitation of HER-2 and HER-3 Receptors

MCF-7 HER-2 cells were examined for association of HER-2 and HER-3 receptors utilizing immunoprecipitation of HER-2 followed by immunoblotting with anti-HER-3 antibody. Cells were grown in 100 mm culture dishes to 80% confluence and in RPMI 1640 media supplemented with 10% FCS. Then, cells were maintained serum-free for 18h prior to the addition of HRG (10 nM) for various times at 37°C. Cell lysates were prepared and total protein concentration was determined as described above. Immunoprecipitations were performed by incubating 0.2 mg lysate protein with 3 μ l of polyclonal anti-HER-2 antibody R60 (Slamon et al., 1987; Slamon et al., 1989a) overnight at 4°C with gentle agitation. Protein A-agarose (BioRad, Richmond, CA) was added to precipitate the antigen-antibody complex and immunoprecipitates were subsequently washed four times in lysis buffer prior to electrophoresis as described above. Proteins were then transferred to nitrocellulose and immunoblotting was performed using anti-HER-3 antibody (Transduction Laboratories, Lexington, KY) as noted above.

Cell Homogenization and Subcellular Fractionation

MCF-7 cells were disrupted in 9 volumes of homogenization media in a Dounce homogenizer with a ball-shaped Teflon pestle using controlled homogenization methods as described (Pietras & Szego, 1979; Pietras & Szego, 1984). Subcellular fractions including nuclear, mitochondria-lysosome, microsomal and particle-free supernatant (cytosol) were prepared by methods previously described (Pietras & Szego, 1979). Activity of 5'-nucleotidase (EC 3.1.3.5) and levels of DNA and protein in the several cell fractions were determined by methods described elsewhere (Pietras & Szego, 1979; Pietras & Szego, 1984).

Specific Binding of Estradiol and Progesterone in Breast Cells

Specific binding of [2,4,6,7-³H (N)]-estradiol (91.3 Ci/mmol; Dupont-New England Nuclear, Boston, MA) to whole cells at 22°C and to subcellular fractions at 4°C was determined by methods described in detail elsewhere (Pietras & Szego, 1979). A 100-fold molar excess of unlabelled estradiol-17 β was present with [³H]-estradiol in paired samples for determination of displaceable binding (Pietras & Szego, 1979). Specific binding of [1,2,6,7-³H (N)]-progesterone (107 Ci/mmol; Dupont-New England



Nuclear) was determined using the same experimental approach. Binding data were analyzed by the method of Scatchard (Read et al., 1989; Welshons et al., 1993). In some experiments, cellular accumulation and binding of 4-hydroxy[N-methyl-³H] tamoxifen (85 Ci/mmol; Amersham, Arlington Heights, IL) was assessed using methods reported elsewhere (Pietras & Szego, 1979; Osborne & Fuqua, 1994).

Determination of Estrogen Receptor Transcripts

In selected in vitro studies, cell RNA was isolated 7 days after plating MCF-7 control or MCF-7 HER-2 cells in estrogen-free media and subjected to Northern blot analysis (Slamon et al., 1987; Slamon et al., 1989a; Chazin et al., 1992), with hybridization of the resulting blot with a human ER cDNA (Green & Chambon, 1988). In vivo experiments using athymic mice, MCF-7 HER-2 cells were treated with exogenous HRG at 2 mg/kg sc every other day beginning with the time of cell inoculation. Paired mice were given vehicle control for comparison as described above. At selected times, tumor tissue was harvested and prepared for extraction of total RNA as previously described (Slamon et al., 1987; Slamon et al., 1989a). The resulting RNA was subjected to Northern blot analysis and hybridized with human ER cDNA.

Transient Transfection of Breast Cells with ERE-CAT Reporter Gene Constructs

A reporter plasmid containing a palindromic ERE and the chloramphenicol acetyltransferase (CAT) gene was used in these studies and is termed ERE-CAT (Lees et al., 1989; Ernst et al., 1991). In brief, an oligonucleotide sequence corresponding to an ERE derived from the vitellogenin A2 promoter of Xenopus laevis (-331 to -295) was cloned into the Xba I site of pBLCAT2. In addition, substitution of the basic reporter plasmid pBLCAT2 for pERE-BLCAT in selected experiments provided a control for specificity of the DNA-binding site in the regulatory sequence of the

reporter gene (CON-ERE). MCF-7 cells used for transfections were cultivated in estrogen-free media for 5 days, then plated in 35-mm wells (3×10^5 cells / well) for 24 h. Prior to transfection, the cultures were washed three times in estrogen-free, serum-free media, and cells were then transfected with 2 μ g DNA of the pERE-BLCAT plasmid using Lipofectamine® (GIBCO) as specified by the manufacturer. After incubation for 8h at 37° C, an equal volume of double-concentrated estrogen-free serum was added, and the medium was then replaced with estrogen-free medium at 24h. To determine ER-independent CAT activation, 2 μ g per 35-mm dish pBLCAT2 was substituted for pERE-BLCAT. Activity of control vehicle, estradiol-17 α (1 nM), estradiol-17 β (1 nM) and heregulin (10nM) was assessed using transfected cells with or without ERE-CAT. In some experiments, the pure antiestrogen, ICI 182,780 (7 α -[9-(4,4,5,5,5-pentafluoropentylsulfinyl) nonyl] estra-1,3,5(10)-triene-3,17 β -diol; generously provided by Zeneca Pharmaceuticals), was used to further assess the specificity of the assay system. Cells were harvested 24h later, and CAT protein was quantitated in cell extracts using a non-radioactive enzyme-linked immunosorbant assay (5 Prime-3 Prime, Boulder, CO) by established procedures (De Maio & Buchman, 1990), with about 50 pg of CAT protein per ml of cell extract found to be the lower limit of detection. CAT reporter activity was normalized for the protein content in each sample.

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Antibody to HER-2/neu receptor blocks DNA repair after cisplatin in human breast and ovarian cancer cells

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Approximately 30% of human breast and ovarian cancers have amplification and/or overexpression of HER-2/neu gene which encodes a cell surface growth-factor receptor. Overexpression of this receptor, p185^{HER-2/neu}, is associated with poor outcome and may predict clinical response to chemotherapy. Antibodies to HER-2/neu receptor have a cytostatic effect in suppressing growth of cells with overexpression of p185^{HER-2/neu}. To elicit a cytoidal effect, therapy with antireceptor antibody was used in combination with the DNA-damaging drug, cisplatin, and this combined treatment produced a synergistic decrease in cell growth. In addition, antibody mediated an increased sensitivity to cisplatin in drug-resistant ovarian carcinoma cells containing multiple copies of HER-2/neu gene. To evaluate the mechanism for this synergy, unscheduled DNA synthesis was measured in cancer cells using incorporation of [³H]thymidine and autoradiography, and formation and repair of cisplatin-induced DNA adducts was also measured. Treatment with cisplatin led to a marked, dose-dependent increase in unscheduled DNA synthesis which was significantly reduced by combined treatment with antireceptor antibody in HER-2/neu-overexpressing cells. Therapy with antibody to HER-2/neu receptor also led to a 35–40% reduction in repair of cisplatin-DNA adducts after cisplatin exposure and, as a result, promoted drug-induced killing in target cells. This phenomenon which we term receptor-enhanced chemosensitivity may provide a rationale for more selective targeting and exploitation of overexpressed growth factor receptors in cancer cells, thus leading to new strategies for clinical intervention.

Introduction

Growth factors and their receptors play pivotal roles in regulation of cell growth and differentiation (Carpenter & Cohen, 1979; Aaronson, 1991). There is now considerable evidence that malignancy arises by a step-wise progression of genetic events that often include the unregulated expression of growth factor receptors or elements of their signaling pathways (Bishop, 1983; Aaronson, 1991; Harris *et al.*, 1992). Among these receptors, the most frequently implicated in human cancers have been members of the epidermal growth factor (EGF) or c-erbB receptor family. The HER-2/neu (c-erbB-2) proto-oncogene encodes a 185 kDa

transmembrane tyrosine kinase, p185^{HER-2/neu}, with homology to EGF receptor (Coussens *et al.*, 1985; Semba *et al.*, 1985). This receptor has oncogenic potential which may be mediated through multiple genetic mechanisms including point mutations in the transmembrane domain (Bargmann *et al.*, 1986), truncation of the extracellular domain or overexpression of the non-mutated proto-oncogene (DiFiore *et al.*, 1987; Hudziak *et al.*, 1987; Yarden & Ullrich, 1988; Aaronson, 1991). Moreover, amplification and/or overexpression of the normal HER-2/neu gene is found in 25–30% of primary human breast and ovarian cancers (Slamon *et al.*, 1987, 1989a) and, less frequently, in other human cancers, including gastric (Park *et al.*, 1989; Kasprzyk *et al.*, 1992) and endometrial (Berchuck *et al.*, 1991) adenocarcinomas. Most importantly, HER-2/neu amplification correlates with a poor prognosis in that patients whose tumors contain this alteration have a shorter disease-free survival as well as a shorter overall survival (Slamon, 1987; Slamon *et al.*, 1989a; Berchuck *et al.*, 1991; Press *et al.*, 1993; Seshadri *et al.*, 1993).

The human HER-2/neu gene is a homologue of the rat c-neu proto-oncogene whose activated form was initially identified as a dominant transforming oncogene in DNA from ethylnitrosourea-induced rat neuroglioblastomas (Shih *et al.*, 1981). Comparison of the transforming neu oncogene sequence with its normal rat proto-oncogene counterpart identified a point mutation in the transmembrane domain which confers increased tyrosine kinase activity to the altered p185^{neu} gene product. This increased activity is believed to be responsible for cell transformation mediated by the mutated gene (Bargmann *et al.*, 1986). To date, no analogous point mutation has been found in the HER-2/neu gene product in human tumors (Slamon *et al.*, 1987, 1989a; Lemoine *et al.*, 1990; Loftis & Gullick, 1992). In contrast, the alteration occurring in human malignant cells is overexpression of a normal gene product which is most frequently but not uniformly due to gene amplification (Slamon *et al.*, 1989a,b; Lemoine *et al.*, 1990). Additionally, overexpression of a structurally-unaltered HER-2/neu gene leads to neoplastic transformation of both NIH3T3 cells (DiFiore *et al.*, 1987; Hudziak *et al.*, 1987) and immortalized, but non-transformed, human breast cells (Pierce *et al.*, 1991; Pietras *et al.*, 1991), indicating that this alteration may play a pathogenic role in promoting tumorigenicity of non-malignant cells.

Monoclonal antibodies against the extracellular domain of the mutated rat *neu* membrane receptor can reversibly suppress tumorigenesis by *neu*-transformed NIH3T3 cells (Drebin *et al.*, 1988). In related studies,

monoclonal antibodies against portions of the extracellular domain of the non-mutated human gene product can specifically inhibit the growth of human breast carcinoma cells overexpressing the HER-2/neu gene product (Hudziak *et al.*, 1989). These antibodies may accomplish their growth-inhibitory effects by blocking a putative autocrine/paracrine growth-stimulatory loop involving p185^{HER-2/neu} receptor (Aaronson, 1991; Harris *et al.*, 1992). Studies with human heregulin, a newly-identified 45 kDa ligand to p185^{HER-2/neu} receptor (Holmes *et al.*, 1992), or other possible p185^{HER-2/neu} ligands (Peles *et al.*, 1992) may help to further define this pathway and its role in malignancy. As with the p185^{HER-2/neu} receptor, overexpression of epidermal growth factor (EGF) receptor is found in several human cancers and is suspected to play a role in tumorigenesis (Yamamoto *et al.*, 1986). Similarly, monoclonal antibodies against the extracellular domain of EGF receptor exhibit significant antitumor activity among cells overexpressing this receptor (Masui *et al.*, 1984). In related studies, Aboud-Pirak *et al.* (1988) identified a poorly understood but probable synergistic tumor cell inhibitory effect between monoclonal antibodies to EGF receptor and the widely-used chemotherapeutic drug, cisplatin. The combined treatment elicited a significant reduction in both the number and size of tumors generated by human epidermoid carcinoma cells which overexpress the EGF receptor. Pursuant to this report, we and others investigated the possibility of a similar effect in human cells overexpressing the p185^{HER-2/neu} receptor. Preliminary data indicated an enhanced cytotoxicity of cisplatin in breast and ovarian cells overexpressing the HER-2/neu gene when grown concomitantly in the presence of antibody specific to an extracellular epitope of the p185^{HER-2/neu} protein (Hancock *et al.*, 1991; Pietras *et al.*, 1991). These studies, however, do not indicate whether the phenomenon is true synergy nor do they provide data regarding the possible mechanism(s) by which it occurs.

We report here a proven synergistic decrease in growth of breast and ovarian cancer cells treated with p185^{HER-2/neu} receptor antibody in combination with cisplatin or carboplatin, drugs used in therapy of human neoplasms (McClay & Howell, 1990; Martin *et al.*, 1992). Maintenance of the integrity of cell DNA by intricate repair pathways is essential to cell survival (Pera *et al.*, 1981; Kwok & Sutherland, 1989). Blockade of cisplatin-induced DNA repair by antireceptor antibody may underlie this effect, offering a new biologic strategy for targeted killing of cells with HER-2/neu overexpression. Elucidation of a pathway for suppression of DNA repair triggered by receptor-specific interactions could have broad significance in cancer therapy. In view of past obstacles to long-term monoclonal antibody therapies in human cancer, an alternative therapeutic use of antireceptor antibodies may be in combination with cytotoxic agents.

Results

Characterization of breast and ovarian cancer cells with HER-2/neu gene overexpression

To investigate the basis of the reported effects between cytotoxic drugs and antireceptor antibodies, we con-

ducted a series of studies with human breast cancer cells containing marked overexpression of p185^{HER-2/neu} receptor as well as with cisplatin-resistant human ovarian carcinoma cells which had either low or high expression of the receptor. SKBR3 breast adenocarcinoma cells, initially derived from a malignant pleural effusion, overexpress the receptor protein based on several-fold amplification of the gene which occurred in the original tumor (Kraus *et al.*, 1987). Ovarian cancer cells 2008 were established from a patient with serous cystadenocarcinoma of the ovary, and the 2008/C13* 5.25 subline (designated C13 here) was obtained by *in vitro* selection for resistance to cisplatin (Andrews *et al.*, 1988). Both the 2008 parental cells and the C13 subline contain a single copy of the HER-2/neu gene and express low levels of the gene product (Pietras *et al.*, 1991). To generate C13 cells containing high expression of the HER-2/neu receptor, multiple copies of full-length human HER-2/neu cDNA (pRVH2) were introduced into parental C13 ovarian cells as previously described (Chazin *et al.*, 1992). Control cells were identically prepared utilizing a control vector devoid of HER-2/neu cDNA (pRVCON). Retrovirally-infected clones were first selected for neomycin resistance and then selected for p185^{HER-2/neu} overexpression by detection of receptor protein using Western blot analyses as shown in Figure 1. As in 2008 ovarian parental cells, minimal expression of p185^{HER-2/neu} protein was found in both the C13 parental and C13pRVCON cells. This is in contrast to marked expression of p185^{HER-2/neu} protein found in C13pRVH2 cells which were engineered to overexpress the gene product (Figure 1). SKBR3 cells naturally overexpress the receptor and were included in these studies for comparison (Figure 1). Independent immunohistochemical analyses confirmed the relative expression levels in the various cell lines and verified a plasma membrane distribution of the receptor (data not shown). Overexpression of the HER-2/neu gene product in C13 cells was found to be associated with pronounced alterations in growth properties. The

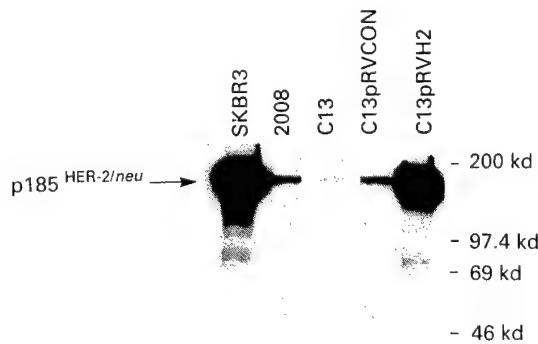


Figure 1 Characterization of p185^{HER-2/neu} receptor expressed in human ovarian and breast carcinoma cells. Using Western blot analyses, SKBR3 breast cancer cells represent a positive control for p185^{HER-2/neu} receptor expression (lane 1 from left) as compared with 2008 ovarian cells (lane 2), C13 ovarian parent cells (lane 3), C13pRVCON cells (lane 4) or C13pRVH2 cells engineered to overexpress p185^{HER-2/neu} protein (lane 5). As indicated in the figure, p185^{HER-2/neu} receptor is a 185 kDa protein. Blots were performed as described elsewhere (Slamon *et al.*, 1989a,b) using anti-HER-2/neu receptor specific antibody

extent of tumor formation by HER-2/neu-overexpressing cells in athymic mice exceeds that of corresponding parental control cells by 3.8-fold after 28 days ($P < 0.001$; data not shown). Given these data, we had available cells with native and molecularly-engineered overexpression of HER-2/neu gene as well as parental control cells containing low levels of the p185^{HER-2/neu} receptor for further investigation.

Effect of HER-2/neu antireceptor antibodies in combination with chemotherapeutic drugs on growth of human cancer cells overexpressing HER-2/neu gene in vitro and in vivo

Several murine monoclonal antibodies reactive to the extracellular domain of the HER-2/neu gene product have been previously characterized (Hudziak *et al.*, 1989; Fendly *et al.*, 1990; Sarup *et al.*, 1991). One of these monoclonal antibodies, 4D5, has been shown to elicit a cytostatic inhibition of the growth of tumor cell lines exhibiting overexpression of the HER-2/neu gene product. Such growth-inhibitory activity may be attributable, in part, to blockade of an autocrine or paracrine growth-stimulatory loop involving the membrane receptor or, alternatively, to direct signaling through the receptor signal transduction pathway. Using *in vitro* assays, we confirm that the 4D5 antibody promotes a marked, dose-dependent reduction in the growth of SKBR3 cells ($P < 0.001$; Figure 2a). A marginal reduction of growth of C13 cells molecularly-engineered to overexpress p185^{HER-2/neu} is also observed with antibody treatment ($P < 0.10$, Figure 3a) while C13 control cells with no overexpression of this protein are not affected by 4D5 (Figure 3a).

The differential sensitivity of SKBR3 and the several ovarian carcinoma cells to cisplatin at concentrations ranging from 0.02–83 μM is shown in Figures 2b and 3b, respectively. As with drug-resistant C13 parental cells, C13pRVH2 cells maintain a cisplatin-resistant phenotype after introduction of the HER-2/neu expression vector (Figure 3b), demonstrating that overexpression of HER-2/neu has no effect on the resistant phenotype in these cells. A more typical dose-response curve for the cisplatin-sensitive 2008 ovarian cells (from which the C13 lines were derived) is also shown in Figure 3b for comparison. When 4D5 antibody is used in combination with cisplatin in SKBR3 (Figure 2b) or C13pRVH2 (Figure 3c) cells, a further and significant suppression of cell proliferation occurs ($P < 0.001$). The cisplatin concentration at which a 50% reduction in cell proliferation occurs (IC_{50}) in the absence vs the presence of 4D5 antibody changed from sixfold in C13pRVH2 cells to greater than 16-fold in SKBR3 cell groups. Treatment of cells with the combination of cisplatin and isotype control antibody had no greater effect on cell proliferation than cisplatin alone ($P > 0.40$). These dose-effect relationships were evaluated further using the method of Chou & Talalay (1984). As required in the latter median-effects approach, cells are grown in the presence of increasing concentrations of antibody or cisplatin alone, and with both agents maintained in a fixed-molar ratio (cf. Figures 2 and 3). Analyses of these data show a synergistic interaction between 4D5 antibody and cisplatin *in vitro* in SKBR3 breast carcinoma cells, with a Combination Index $_{50} < 0.5$. These effects were also found

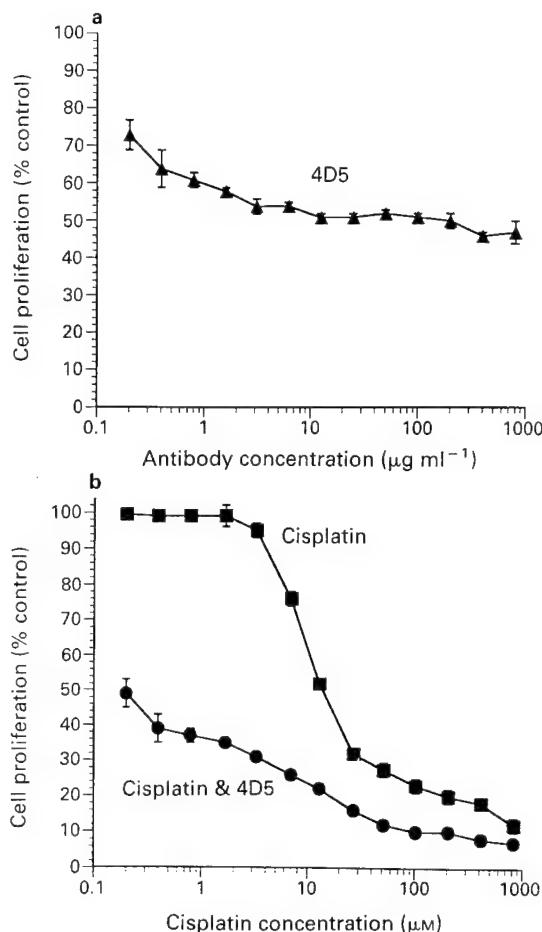


Figure 2 Sensitivity of SKBR3 breast carcinoma cells to cisplatin and HER-2/neu antireceptor antibody and synergistic interactions. (a) SKBR3 cell proliferation *in vitro* in the presence of 4D5 antibody at concentrations ranging from 0.2 to 800 $\mu\text{g ml}^{-1}$ [▲; 4D5]. (b) Proliferation of SKBR3 cells was determined in the presence of cisplatin ranging from 0.02 to 83 μM [■; cisplatin]. SKBR3 cells were also treated with 4D5 together with cisplatin, with the combination given at a fixed-molar ratio of 64:1 (cisplatin:4D5). Concentrations of cisplatin ranged from 0.02 to 83 μM and are shown on the abscissa; corresponding conventions of 4D5 ranging from 0.3 nM to 1.3 μM (i.e., 0.05 to 200 $\mu\text{g ml}^{-1}$) in order to maintain a fixed-molar ratio of 64:1 (cisplatin:4D5) with cisplatin were present in this experiment but are not displayed on the abscissa [●; cisplatin & 4D5]. In these studies, drug was added 5 min after antibody. Control experiments were conducted with non-p185^{HER-2/neu} antibodies of the same class and isotype (IgG1) and/or cisplatin vehicle as appropriate

when 4D5 was added in combination with carboplatin in SKBR3 cells ($P < 0.001$; data not shown). Similar analyses of data for C13pRVH2 cells required mathematical extrapolation of the dose required to produce a median effect for treatment with antibody alone (cf. Figure 3a). With the proviso that the latter dose projection is valid, the resulting calculations predict a Combination Index $_{50} < 0.5$ as found with the breast carcinoma cells. To confirm the relative receptor-dependent specificity of this phenomenon, C13pRVCON cells which do not overexpress HER-2/neu proto-oncogene were treated with identical antibody/drug combinations, and no apparent synergistic decrease in cell growth was observed (Figure 3). Similarly, the IC_{50} ratio in the absence vs the presence of 4D5 antibody was 0.97 in C13pRVCON cells.

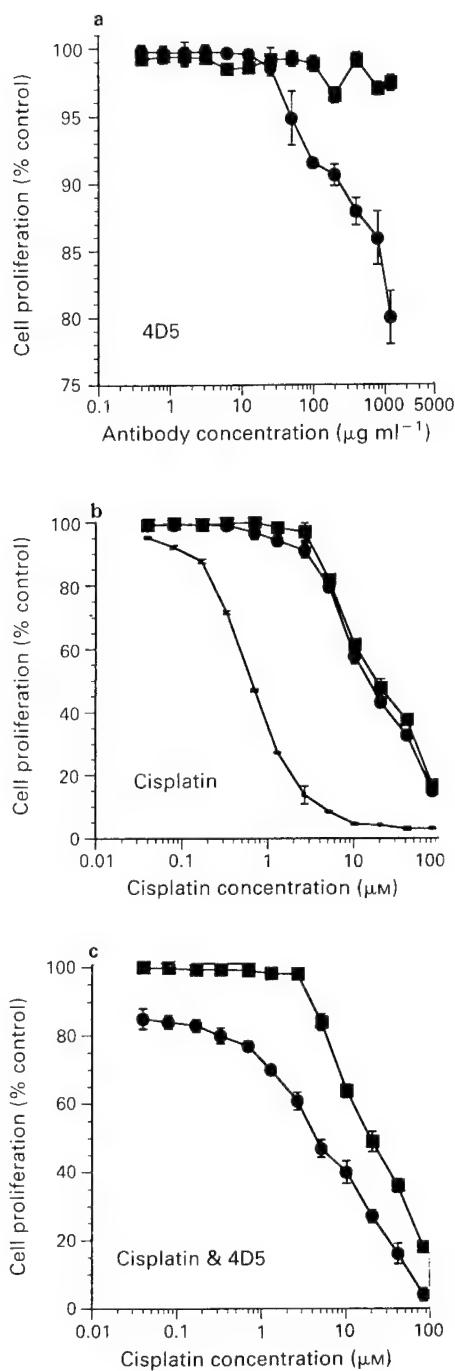


Figure 3 Sensitivity of ovarian carcinoma cells to cisplatin and HER-2/neu antireceptor antibody and synergistic interactions. (a) C13 ovarian cell proliferation *in vitro* in the presence of 4D5 antibody ranging from $0.4 \mu\text{g ml}^{-1}$ to 1.2 mg ml^{-1} [■; C13pRVCON/4D5; ●; C13pRVH2/4D5]. (b) Proliferation of cisplatin-resistant C13 cells was determined in the presence of cisplatin ranging from 0.04 to $83 \mu\text{M}$ [■; C13pRVCON/cisplatin; ●; C13pRVH2/cisplatin]. For comparison, the growth response of cisplatin-sensitive 2008 cells in the presence of the same dose range of cisplatin are also shown (reduced points, 2008/cisplatin). (c) C13 cell groups were also treated with 4D5 together with cisplatin, with the combination given at a fixed molar ratio of 64:1 (cisplatin:4D5). Concentrations of cisplatin ranged from 0.04 to $83 \mu\text{M}$ and are shown on the abscissa. Corresponding concentrations of 4D5 ranging from 0.7 nM to $1.3 \mu\text{M}$ (i.e., 0.1 to $200 \mu\text{g ml}^{-1}$) were also present in order to maintain a fixed-molar ratio of 64:1 (cisplatin:4D5), but these values are not displayed on the abscissa [■, C13pRVCON/cisplatin & 4D5; ●, C13pRVH2/cisplatin & 4D5]. In these experiments, drug was added 5 min after antibody. Control experiments were conducted with non-HER-2/neu antibodies of the same class and isotype (IgG1) and/or cisplatin vehicle as appropriate.

To confirm and extend the *in vitro* studies of the synergistic effect between 4D5 and cisplatin, the combination was tested for inhibition of growth of subcutaneous tumor xenografts in athymic nude mice. Growth of ovarian C13pRVH2 cells was monitored in animals treated with either 4D5 alone, isotype control antibody, cisplatin alone or a combination of antibody and cisplatin (Figure 4). Previous pharmacokinetic studies using antibody 4D5 have been presented and demonstrate that single-dose therapy with this monoclonal antibody leads to maintenance of a significant serum antibody concentration of several days duration (DeSantes *et al.*, 1992). Treatment of tumor-bearing mice with a single dose of 4D5 caused a concentration-dependent inhibition of tumor growth at antibody doses ranging from 25 – 150 mg kg^{-1} ($P < 0.01$; Figure 4a). Growth inhibition by antibody alone is cytostatic since tumor growth resumes by 21–28 days after the antibody dose. Treatment of mice with cisplatin alone at doses of 6 – 9 mg kg^{-1} , but not at 3 mg kg^{-1} , elicited a similar dose-dependent decline in tumor growth ($P < 0.01$; Figure 4b). Therapy of tumor-bearing animals with 4D5 in combination with cisplatin results in a significant and marked inhibition of tumor growth exceeding the effect of either agent given alone ($P < 0.005$; Figure 4c). Analyses of these dose-effect relationships by the method of Chou & Talalay (1984) again show a substantial synergistic interaction between the 4D5 antibody and cisplatin *in vivo*, with a Combination Index $_{50} = 0.16$, further substantiating the superior therapeutic effect of combined therapy. Data presented in Figure 5 indicate that the benefit of this treatment is sustained over a 6 week period after one dose of combined therapy. Cisplatin and antireceptor antibody administered together elicit a logarithmic reduction in ovarian tumor size as compared to that observed with cisplatin or antibody given as single agents ($P < 0.001$).

Effect of HER-2/neu antireceptor antibodies in combination with chemotherapeutic drugs on unscheduled DNA synthesis

After demonstrating both *in vitro* and *in vivo* a clear synergistic effect of the combination of 4D5 and cisplatin in HER-2/neu-overexpressing cells, experiments were designed to evaluate the possible mechanism(s) for this phenomenon. To determine if the synergistic increase in drug-mediated cytotoxicity occurring with antibody was a result of an increase in cellular accumulation of cisplatin, we conducted independent experiments using methods previously described (Andrews *et al.*, 1988). These studies showed no significant effect of 4D5 at doses up to $100 \mu\text{g ml}^{-1}$ on accumulation of $83 \mu\text{M}$ [^{14}C]carboplatin by SKBR3 cells over 3 to 24 h (data not shown), indicating that the synergistic effect does not appear to occur by altered cellular accumulation of this chemotherapeutic drug.

DNA repair is well known to play an important role in the recovery of cells from the toxicity of cisplatin (Pera *et al.*, 1981; Scanlon & Kashani-Sabet, 1988; Whitaker, 1992; Zhen *et al.*, 1992). To evaluate whether changes in DNA repair mechanisms might be a potential explanation for the synergistic interaction of anti-receptor antibody and platinum-derived drugs, we measured unscheduled DNA synthesis (Trosko &

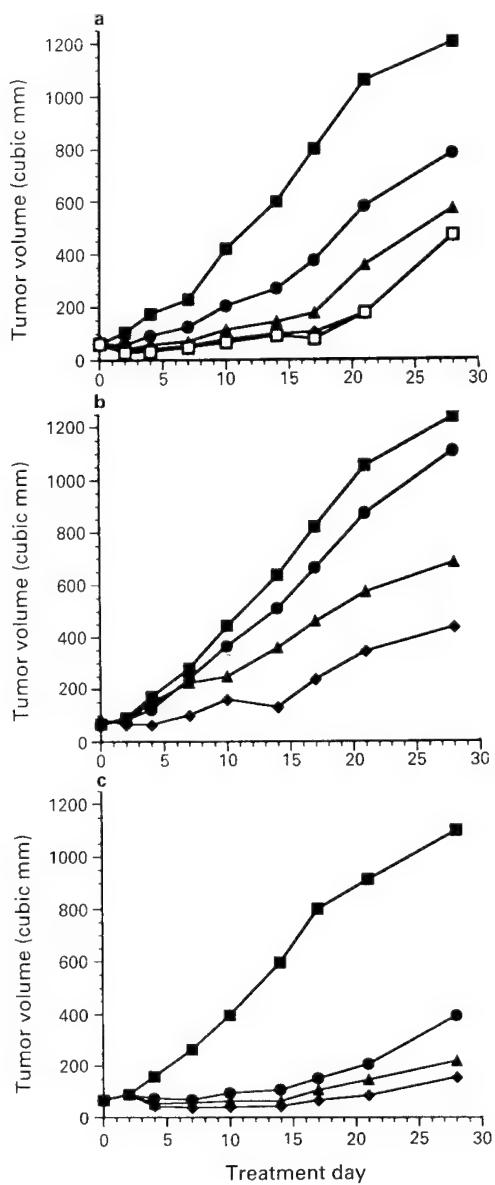


Figure 4 Growth of C13pRVH2 ovarian cancer cells in nude mice and effect of treatment with 4D5 antibody, cisplatin and antibody/drug combinations. (a) The growth of C13pRVH2 tumors is shown in the presence of control (■) or 4D5 antireceptor antibody at 5 mg kg⁻¹ (●), 25 mg kg⁻¹ (▲), 75 mg kg⁻¹ (◆), or 150 mg kg⁻¹ (□) given by intraperitoneal injection on day 0. (b) The growth of C13pRVH2 tumors is shown in the presence of control (■) or cisplatin at 3 mg kg⁻¹ (●), 6 mg kg⁻¹ (▲), or 9 mg kg⁻¹ (◆) given by i.p. injection on day 0. (c) Finally, the growth of C13pRVH2 tumors is shown on treatment with control (■) or 4D5 antibody/cisplatin combinations at 2.5/1.5 mg kg⁻¹ (●), 5.0/3.0 mg kg⁻¹ (▲) and 10.0/6.0 mg kg⁻¹ (◆) respectively. Antireceptor antibody 4D5 was given i.p. at day 0, and cisplatin was given 18 h later. Ovarian cells were injected subcutaneously at 5×10^7 cells per animal. After 1 week, mice were randomized on day 0 to groups of 3–4 animals on the basis of body weight and tumor nodule size. Animals received either IgG1 antibody control (150 mg kg⁻¹), 4D5 antibody, cisplatin or a combination treatment (see Materials and methods for additional details)

Yager, 1974; Williams, 1977) induced by cisplatin in SKBR3 and C13 cells (Figure 6a). As expected, treatment of SKBR3 cells with cisplatin alone provoked a significant increase in unscheduled DNA synthesis to 2.1-times the control level as determined by [³H]thymidine incorporation into DNA. These data indicate an

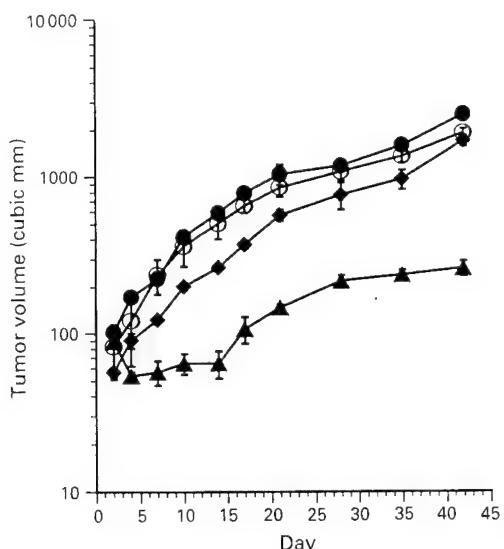


Figure 5 Growth of C13pRVH2 ovarian cancer cells in nude mice over 6 weeks and effect of a single treatment with 4D5 antibody, cisplatin or antibody/drug combination. The growth of C13pRVH2 tumors is shown in the presence of control (●), 4D5 antireceptor antibody at 5 mg kg⁻¹ (◆), cisplatin at 3 mg kg⁻¹ (○), or 4D5 antibody/cisplatin combination at 5.0/3.0 mg kg⁻¹ (▲), respectively. Antireceptor antibody 4D5 was given i.p. at day 0, and cisplatin was given 18 h later as in Figure 3. Ovarian cells were injected subcutaneously at 5×10^7 cells per animal. After 1 week, mice were randomized on day 0 to groups of 3–4 animals on the basis of body weight and tumor nodule size

active DNA repair apparatus in these cells ($P < 0.001$; Figure 6a). Treatment with 4D5, however, almost completely blocked this cisplatin-induced increase in DNA synthesis. To confirm that this phenomenon was specifically due to HER-2/neu overexpression, it was also tested in cisplatin-resistant C13 cells with and without overexpression of HER-2/neu. In these studies, C13pRVH2 cells, but not C13 control cells, exhibited an antibody-induced suppression of DNA repair ($P < 0.01$; Figure 6a). Carboplatin at 34 μ M also promoted unscheduled DNA synthesis in SKBR3 and C13 cells ($P < 0.01$). This effect was similarly blocked by combined treatment with 4D5 (200 μ g ml⁻¹) in cells overexpressing HER-2/neu but not in control cells (data not shown). To confirm and extend these observations, an alternative measure of unscheduled DNA synthesis was performed. Autoradiographic localization of silver grains due to [³H]thymidine uptake in cell nuclei provided independent data demonstrating that this phenomenon does indeed occur. Cisplatin, but not 4D5, enhances unscheduled DNA synthesis in both SKBR3 and C13 cells (Figure 6b). Again, this drug-induced effect is blocked by combined treatment with antireceptor antibody in cells overexpressing HER-2/neu but not in the C13 control cells, confirming that 4D5 interferes with DNA repair only in those cells overexpressing the p185^{HER-2/neu} receptor.

Effect of HER-2/neu antireceptor antibody on formation and repair of cisplatin-induced DNA adducts in the DNA of cisplatin-sensitive and -resistant human ovarian cancer cells

Since measures of unscheduled DNA synthesis provide only an indirect assessment of actual DNA repair, we

sought to obtain direct data on the formation and removal of cisplatin-induced lesions in total genomic DNA of ovarian carcinoma cells (Table 1). Cisplatin-sensitive parental (2008) and cisplatin-resistant C13 ovarian carcinoma cells with overexpression

(C13pRVH2) or normal expression (C13pRVCON) of the HER-2/neu gene were each treated with 200 μM cisplatin for 1 h at 37°C, washed and then harvested at 0 or 24 h after the initial cisplatin treatment. To test the effect of antireceptor antibody, cells were first exposed to 4D5 (200 $\mu\text{g ml}^{-1}$) or control solution for 6 h prior to cisplatin treatment. After cisplatin exposure and cell washing, 4D5 was maintained in the culture medium at 200 $\mu\text{g ml}^{-1}$ for the repair times indicated in Table 1. Table 1 shows data for the formation and removal of cisplatin-DNA adducts from the genomic DNA of cells treated with and without antireceptor antibody in three separate experiments. The initial frequency of cisplatin lesions in the parental 2008 cells is six to seven times higher than in the C13 resistant cells as found by others (Zhen *et al.*, 1992). This is consistent with the cisplatin-resistant phenotype of the C13 cells. In the absence of antireceptor antibody, removal of cisplatin-induced DNA adducts at 24 h, a direct measure of DNA repair, occurs at similar rates in the cisplatin-resistant cells (C13pRVH2 and C13pRVCON). Both the 2008 and C13pRVCON cells contain low expression of HER-2/neu, and combined therapy with 4D5 antibody and cisplatin had no significant change in the rate of cisplatin-induced DNA adduct removal at 24 h. However, treatment of HER-2/neu-overexpressing C13pRVH2 cells with antireceptor antibody prior to cisplatin promoted a significant reduction in the extent of DNA repair to 64% of that found in cells not treated with antibody ($P < 0.05$). Moreover, the actual rate of drug-DNA adduct repair found in the cisplatin-resistant C13pRVH2 cells treated with the antireceptor antibody approached that found in the cisplatin-sensitive 2008 cells, indicating that the cisplatin-resistant phenotype can be reversed by this combined therapy.

To assure that the effect noted above was not attributable solely to an excessive cisplatin concentration, C13pRVH2 cells were also exposed for 1 h to cisplatin at 10 μM , a dose closer to the IC_{50} for these cells. Otherwise, this experimental series was conducted using a treatment protocol as outlined above, but determination of platinum levels in cellular material was done by a sensitive method of inductively-coupled plasma atomic emission spectrometry (Dominici *et al.*, 1989). The results show that repair of cisplatin-DNA adducts at 24 h average 71% in C13pRVH2 cells treated with low-dose cisplatin and control solution and 42% in C13pRVH2 cells exposed to low-dose

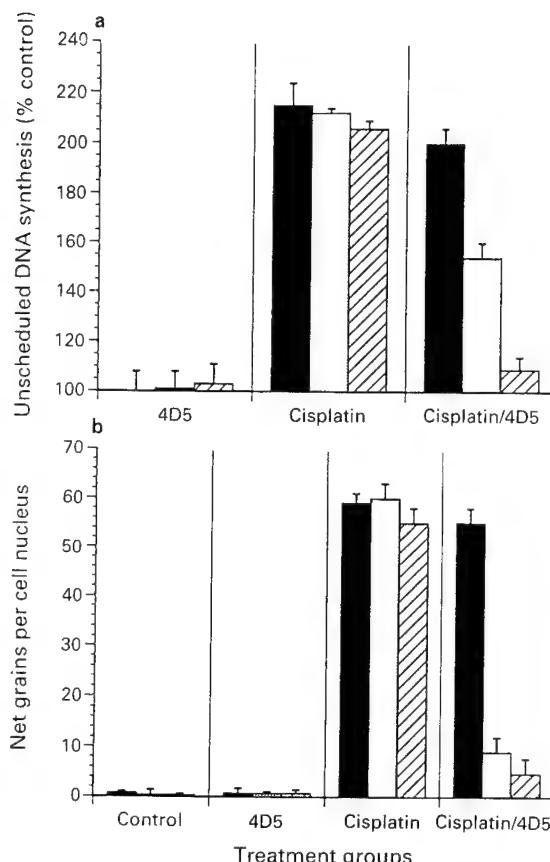


Figure 6 DNA repair (unscheduled DNA synthesis) in human breast and ovarian carcinoma cells after treatment with 4D5, cisplatin (DDP) or 4D5/cisplatin (4D5/DDP) combinations. (a) Unscheduled DNA synthesis was measured in C13pRVCON (black bar), C13pRVH2 (white bar), and SKBR3 (hatched bar) cells in the presence of 4D5 (200 $\mu\text{g ml}^{-1}$), cisplatin (10 μM) or 4D5/cisplatin (200 $\mu\text{g ml}^{-1}$ and 10 μM , respectively). (b) Measurement of DNA repair by autoradiographic localization of radioactive thymidine was tabulated in C13pRVCON (black bar), C13pRVH2 (grey bar), and SKBR3 (hatched bar) cells. Counts of developed silver grains in the photographic emulsion overlying cell nuclei were compared after treatment with 4D5 (200 $\mu\text{g ml}^{-1}$), cisplatin (DDP; 10 μM), 4D5/carboplatin (200 $\mu\text{g ml}^{-1}$ and 10 μM , respectively) or control (CON) solutions. Unscheduled DNA synthesis was determined as described in Materials and methods

Table 1 Effect of 4D5 antibody on formation and repair of cisplatin adducts in the genome in sensitive and resistant human ovarian cancer cells with differential expression of HER-2/neu proto-oncogene*

Therapy	Cisplatin-sensitive cells 2008			Cisplatin-resistant cells C13pRVCONTROL			C13pRVHER-2		
	Repair time (h)	Pt-Adducts (pg/ $\mu\text{g DNA}$)	% Repair	Pt-Adducts (pg/ $\mu\text{g DNA}$)	% Repair	Pt-Adducts (pg/ $\mu\text{g DNA}$)	% Repair		
Without antibody	0	40.4 \pm 2.8		6.1 \pm 2.5		6.4 \pm 2.6			
	24	26.4 \pm 2.4	35	2.3 \pm 1.7	62	2.7 \pm 1.1	58		
With antibody	0	43.6 \pm 2.7		5.7 \pm 1.7		7.2 \pm 2.7			
	24	29.2 \pm 2.5	33	2.1 \pm 1.5	63	4.5 \pm 1.7	38†		

*Cells were treated with 200 μM cisplatin (Pt) for 1 h in the presence or absence of anti-HER-2/neu receptor antibody 4D5 as outlined in the text. The time indicated is hours elapsed after cisplatin treatment. Percent repair was estimated as described elsewhere (Jones *et al.*, 1991; Zhen *et al.*, 1992) using Pt-adduct counts at 0 and 24 h as shown here. The latter counts were corrected for DNA replication as noted in the text. †Significantly different from control at $P < 0.05$.

cisplatin and 4D5 monoclonal antibody (data not shown; $n = 2$). Collectively, these findings demonstrate that removal of cisplatin adducts from the genome of HER-2/neu-enriched cells is reduced by about 35–40% in the presence of antireceptor antibody.

Discussion

The data presented in this report provide direct evidence of a true synergistic antitumor effect of combined treatment of HER-2/neu-overexpressing cancer cells with monoclonal antibodies to the p185^{HER-2/neu} receptor and the DNA-damaging drug, cisplatin. The increased killing of the overexpressing cells cannot be attributed merely to the additive effect of the two drugs acting independently. Moreover, the high degree of synergistic interaction observed *in vivo* is likely to translate to real therapeutic gains (Berenbaum, 1989). Other studies show similar cytotoxicity with antibodies to EGF receptor and cisplatin in human epidermoid carcinoma xenografts that overexpress EGF receptor (Aboud-Pirak *et al.*, 1988). Subsequent studies demonstrated an increased killing of breast and ovarian cells grown continuously in the presence of cisplatin and antibody to the extracellular domain of p185^{HER-2/neu} receptor (Hancock *et al.*, 1991; Shepard *et al.*, 1991; Pietras *et al.*, 1992). These results are confirmed in the present work. It is important to note that interpretation of such data on combined-drug effects often does not weigh the therapeutic advantage of a proposed treatment (Berenbaum, 1989). Thus, drug combinations which elicit little additional cell killing, regardless of any formal mathematical demonstration of synergy, may have little therapeutic application. True therapeutic value likely requires drug combinations which generate a logarithmic increase in cell killing or induce tumor remissions which could not be achieved by independent treatment at sublethal doses (Berenbaum, 1989). The data presented in this report meet these stringent criteria for a therapeutic advantage of antireceptor antibody and a DNA-damaging drug.

Binding of certain growth factors to their cognate receptors has been reported to modulate cellular sensitivity to drugs and to physical agents. Incubation of human ovarian carcinoma cell lines with EGF has been found to increase sensitivity of these cells to the cytotoxic effects of cisplatin (Christen *et al.*, 1991a,b). Similarly, EGF is reported to enhance the sensitivity of human squamous carcinoma cells to radiation therapy (Kowk & Sutherland, 1989). Treatment of human neuroblastoma cells with NGF reduces DNA repair induced by either ultraviolet radiation or benzo(a)pyrene (Jensen & Linn, 1988). On the other hand, basic fibroblast growth factor (bFGF) promotes repair of radiation-induced damage in endothelial cells. Anti-bFGF antibodies block growth factor binding to its receptor and simultaneously present repair of radiation damage to cellular DNA (Haimovitz-Friedman *et al.*, 1991). Despite such observations, specific mechanisms mediating the relationship between growth factor receptors and DNA repair pathways are unknown. It may be important to consider that EGF receptor has been reported to be a bifunctional enzyme. In addition to the intrinsic tyrosine kinase activity activated by

binding of ligand, this receptor also exhibits a DNA repair enzyme activity (Mroczkowski *et al.*, 1984). Further, EGF receptor is associated with modulation of cell progression from the G₂ phase of the cell cycle to mitosis, a period of active DNA repair prior to cell division (Kinzel *et al.*, 1990). A protein kinase involved in regulation of cell division in budding yeast, HRR25, is also associated with repair of damaged DNA (Hoekstra *et al.*, 1991), and certain DNA repair enzymes in prokaryotes derive from membrane transport proteins (Doolittle *et al.*, 1986). The potential biologic significance of such DNA repair capability in an erbB family receptor protein remains to be determined.

Although the downstream events due to cisplatin therapy (Masuda *et al.*, 1988; Whitaker, 1992; Zhen *et al.*, 1992) or to interactions between HER-2/neu antireceptor antibody and the receptor (Drebin *et al.*, 1988; Sarup *et al.*, 1991) are incompletely characterized, evidence is developing to suggest that antibodies directed against the HER-2/neu receptor can disrupt specific programs of gene expression and result in inhibition of growth (Drebin *et al.*, 1988; Hudziak *et al.*, 1989; Shepard *et al.*, 1991) or modulate the sensitivity to DNA-damaging drugs such as those studied here. Cisplatin tends to produce intrastrand adducts and interstrand crosslinks in DNA (Zhen *et al.*, 1992). A significant role of DNA repair in the recovery of cells from the toxicity of drugs such as cisplatin has been established by several different lines of evidence (Pera *et al.*, 1981; Scanlon & Kashani-Sabet, 1988; Perez *et al.*, 1991). In cells resistant to such DNA-damaging drugs, increased levels of various DNA repair enzymes have been detected, suggesting this as a possible mechanism of resistance (Pera *et al.*, 1981; Scanlon & Kashani-Sabet, 1988; Whitaker, 1992). It is also well established that DNA repair-deficient cells exhibit a markedly enhanced sensitivity to ionizing radiation and alkylating agents (Perez *et al.*, 1991; Whitaker, 1992). Such findings are consistent with the present results which indicate that HER-2/neu antireceptor antibody can amplify the cytotoxicity of a DNA-damaging drug by inhibition of DNA repair.

A further aspect of the present findings is the possibility that overexpression of HER-2/neu proto-oncogene is intrinsic to genesis of cellular resistance to DNA-damaging agents. The involvement of other oncogenes in the development of chemotherapeutic drug resistance has been proposed by Scanlon *et al.* (1989), and evidence in support of this hypothesis has been generated (Sklar, 1988; Ishonishi *et al.*, 1991). The potential role of c-erbB proto-oncogenes in modulation of chemotherapeutic drug sensitivity has been suggested from retrospective analysis of results of several therapeutic clinical studies and, if correct, could have important implications in patient management and treatment decisions (Wright *et al.*, 1989; Allred *et al.*, 1992; Gusterson *et al.*, 1992; Klijn *et al.*, 1992; Tsai *et al.*, 1993). The present work, however, does not demonstrate further potentiation of cisplatin resistance *in vitro* in ovarian cells following the introduction of excess HER-2/neu gene expression. However, this cell line was selected for drug resistance prior to overexpression of the oncogene and may not represent a good model to test this important question. Additional studies with drug-sensitive cells are required to address the potential role of specific oncogenes in drug resis-

tance of cancer cells, and such studies are currently underway in our laboratory.

This report demonstrates that HER-2/neu receptor antibody interferes with DNA repair induced by cisplatin and, as a result, promotes drug cytotoxicity in target cells in a synergistic fashion. We are confident that this phenomenon is biologically meaningful in that we have demonstrated it in more than one cell line, across two different types of epithelial cells and in both naturally and molecularly-engineered HER-2/neu-overexpressing cells. Such an approach to suppression of DNA repair in targeted cell populations (i.e., using antireceptor antibodies) may offer an important new modality against breast and ovarian cancer cells with overexpression of p185^{HER-2/neu} receptor. The potential specificity of the therapeutic use of anti-HER-2/neu antibodies in altering DNA repair in such a way as to increase the sensitivity of HER-2/neu-overexpressing cells to both chemical and physical DNA-damaging agents is supported by reports showing little to no reactivity of such antibodies with most normal human tissues (Press *et al.*, 1993). The synergistic therapeutic phenomenon demonstrated in these studies, which we term receptor-enhanced chemosensitivity, may provide a new approach to target and exploit overexpressed growth factor receptors in a variety of malignant cells, and may lead to new, biologically-based therapeutic strategies for clinical intervention.

Materials and methods

Cell lines and cell culture

Human ovarian 2008 cells were established from a patient with serous cystadenocarcinoma of the ovary (DeSaia *et al.*, 1972), and the 2008/C13*5.25 subline (designated C13 here) was obtained by selection *in vitro* for resistance to cisplatin (Andrews *et al.*, 1988). In addition, the human breast carcinoma cell line, SKBR3, was obtained from American Type Culture Collection (Rockville, MD). All cells were routinely cultured in RPMI medium 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM freshly added glutamine and 1% penicillin G-streptomycin-fungizone solution (Irvine Scientific, Santa Ana, CA).

Transfections and amplification/overexpression of human HER-2/neu gene in human ovarian cells

Human ovarian cells 2008 and C13, with low levels of expression of the HER-2/neu gene, were transfected with full-length cDNA of human HER-2/neu gene. The latter was cloned from primary human breast tissue and characterized previously in our laboratory (cf. Slamon *et al.*, 1989a,b). The vector for introduction of HER-2/neu gene into human cells contains the full-length human HER-2/neu gene which was ligated into the replication-defective retroviral expression vector, pLXSN (cf. Miller & Rosman, 1989). This was achieved by ligating a 3.8 kb NcoI to MstII fragment containing the full HER-2/neu coding sequence, but no polyadenylation signal, into an amphotrophic retroviral expression vector with a Moloney murine leukemia virus (MMLV) promoter, a neomycin phosphotransferase gene and a packaging signal, but devoid of viral protein coding sequences; thus rendering the virus replication-defective. The pLXSN construct has an extended packaging signal for high virus titre as well as a mutated gag start codon and a shortened envelope region to decrease the risk of helper virus generation (Miller & Rosman, 1989; Chazin *et al.*, 1992). The resulting expression vector is termed pRVH2. Virus-producing cells were pre-

pared by a transient rescue procedure as described before (Miller & Rosman, 1989; Chazin *et al.*, 1992). As noted above, this vector also contains a neomycin resistance gene (neomycin phosphotransferase) which confers cellular resistance to the aminoglycoside antibiotic G418, thus allowing selection of primary infectants. The pLXSN vector devoid of HER-2/neu sequences but containing the neomycin phosphotransferase gene was packaged in an identical fashion and served as a retroviral control in appropriate experiments (designated pRVCON).

Ovarian carcinoma cells were infected as described (Miller & Rosman, 1989; Chazin *et al.*, 1992). In brief, 0.1 ml of virus-containing supernatant from helper-free producer clones was used to infect 1×10^6 cells in the presence of polybrene ($4 \mu\text{g ml}^{-1}$). After 24 h, cells were split 1:10 into media containing $500-750 \mu\text{g ml}^{-1}$ of G418 for selection. Infectants with and without the HER-2/neu gene were obtained and cloned by use of cloning rings and limiting dilutional cloning as described by Chazin *et al.* (1992). All cell lines established by this method of gene transfer were characterized at the RNA, protein and immunohistochemical level for expression level of HER-2/neu gene by previously described methods (Slamon *et al.*, 1989a; Pietras *et al.*, 1991; Chazin *et al.*, 1992).

Cell proliferation assay

Aliquots of 5×10^3 cells were plated in triplicate in 96-well microdilution plates. Following cell adherence, experimental or control media were added. After incubation at 37°C for various time periods, plates were washed twice with normal saline and then stained with crystal violet (0.5% in methanol). Plates were gently washed three times in distilled water and allowed to dry. The crystal violet was dissolved in 0.1 ml Sorenson's Buffer, and the plates were analysed in an ELISA plate-reader at 540 nm wavelength. The intensity of staining correlates with extent of cell proliferation as reported elsewhere (Hudziak *et al.*, 1989; Pietras *et al.*, 1991).

Tumor formation in nude mice

Ovarian cells were injected subcutaneously at 5×10^7 cells per animal in the mid-back region of female Swiss nude mice (12 weeks old). After 1 week, animal body weights and tumor nodule dimensions were measured, and mice were randomized on day 0 to groups of 3–4 animals on the basis of body weight and tumor nodule size. Mice were treated via intraperitoneal injection. Animals received either an isotype-matched IgG1 antibody control (150 mg kg^{-1}), 4D5 antibody, cisplatin or a combination treatment at the start of the experiment. Mice receiving combination treatments were given injections of cisplatin 18 h after injection of antibody. Prospective tumor nodules were monitored by micrometer measurements, with tumor volume calculated as the product of length \times width \times height. Tumors formed were analysed for HER-2 expression by immunohistochemical methods as described in detail elsewhere (Slamon *et al.*, 1989a,b).

Monoclonal antibodies

Monoclonal antibody 4D5 is directed to an extracellular epitope of p185^{HER-2/neu} receptor and was prepared as described in detail elsewhere (Fendly *et al.*, 1990). Control experiments were conducted with nonspecific IgG of the same class and isotype to verify the specificity of any observed effects.

Unscheduled DNA synthesis

Unscheduled DNA synthesis (UDS), DNA repair which is nonsemiconservative in nature, was determined by estab-

lished methods (Trosko & Yeager, 1974; Montine & Borch, 1988). Cell monolayers were preincubated with or without antibody in arginine-deficient, reduced serum (0.5%) media for 5 h, followed by exposure to hydroxyurea for 1 h. Then, cells were treated with cisplatin (in the presence of hydroxyurea) for 1 h and finally incubated with [³H]thymidine and hydroxyurea for 3 h. Cell groups were harvested, and cellular DNA bound to glass fiber filters was collected for liquid scintillation counting of [³H]thymidine incorporation/group. Measurement of UDS by the alternate approach of autoradiography following radioactive thymidine exposure (i.e., by counting developed silver grains in the photographic emulsion overlying cell nuclei) was also used for independent confirmation of findings. In autoradiography experiments, cells were preincubated and treated as above, then incubated with [³H]thymidine (10 µCi ml⁻¹) and hydroxyurea for 16 h and processed as described elsewhere (Williams, 1977; Butterworth *et al.*, 1987).

Detection of genomic cisplatin adducts

Cells were cultivated *in vitro* to 60 to 70% confluence. For 12 h prior to the start of the experiment, cells were labeled with [³H]thymidine at 0.1 µCi ml⁻¹ in order to provide a correction factor for cellular replication during the course of the experiment (Jones *et al.*, 1991). Thereafter, cells were incubated in fresh medium with 4D5 antibody at 200 µg ml⁻¹ or control solution for 6 h. The cells were then exposed to 200 µM cisplatin (freshly made) for 1 h, washed in cisplatin-free media and harvested at 0 and 24 h after the cisplatin treatment. Cells treated with or without 4D5 antibody were maintained in the same media after removal of the drug. Harvested cells were pelleted and stored at -20°C until DNA isolation. DNA was isolated and prepared as described before (Bohr & Okumoto, 1988; Zhen *et al.*, 1992). Total platinum content was assessed by atomic absorption spectrometry using a Perkin-Elmer Zeeman spectrometer (cf Zhen *et al.*, 1992). In selected experiments with low-dose 20 µM cisplatin, cell DNA was obtained as above, but total platinum content was measured by inductively-coupled plasma atomic emission spectrometry for sensitive determina-

tion of cisplatin content in cellular DNA samples (cf. Dominici *et al.*, 1989).

Data analysis

The nature of the interaction between monoclonal antibody to p185^{HER-2}^{neu} receptor (4D5) and cisplatin was investigated using median-effects analysis (Chou & Talalay, 1984). Cells were grown in the presence of increasing concentrations of antibody or cisplatin alone and with both agents maintained in a fixed molar ratio. It was verified that each agent alone and in combination produced a linear median-effect plot in order to demonstrate that the dose-response relationships followed the basic mass-action principle. Moreover, it was established that slopes of the median-effect plots for each agent alone and in combination did not differ significantly from each other, indicating that they were not acting by independent mechanisms (i.e., they were not mutually exclusive). The combination index as derived from the latter plots provides a quantitative measure of the extent of drug interactions. A value of one indicates that the drugs are simply additive; a value of >1 indicates antagonism; and a value of <1 indicates synergy (Chou & Talalay, 1984).

For statistical analysis, one-way analysis of variance (ANOVA) was conducted on tumor size data at each time point as appropriate. Average tumor size or relative cell proliferation data in each treated group was compared to that in the appropriate control group via a *t* test using conventional methods (Campbell, 1967). Data are presented as mean with standard error.

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